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(54) Title: IGF/IGFBP COMPLEX FOR PROMOTING BONE FORMATION AND FOR REGULATING BONE REMODELING

## (57) Abstract

IGF and IGFBP are administered to stimulate new bone formation in subjects with bone loss due to bone marrow disorders, connective tissue disorders, drugs, pregnancy, lactation, chronic hypophosphatemia, hyperphosphatasia, insulin-dependent diabetes mellitus, anorexia nervosa, cadmium poisoning, juvenile osteoporosis, Paget's disease of bone, osteoarthritis and periodontal disease. IGF-I and IGFBP-3 are optionally combined with agents that inhibit bone resorption.

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5           IGF/IGFBP COMPLEX FOR PROMOTING BONE FORMATION  
              AND FOR REGULATING BONE REMODELING

Technical Field

10           The instant invention relates generally to  
polypeptide factors and their use in bone growth and  
maturation. Specifically, the invention relates to the  
use of a complex of insulin-like growth factor (IGF) and  
insulin-like growth factor binding protein (IGFBP) with  
or without an inhibitor of bone resorption to stimulate  
15 bone formation.

Background Art

          Growth factors are polypeptides which stimulate  
a wide variety of biological responses (e.g., DNA  
20 synthesis, cell division, cell differentiation,  
expression of specific genes, etc.) in a defined  
population of target cells. A variety of growth factors  
have been identified including transforming growth  
factor- $\beta$ 1 (TGF- $\beta$ 1), TGF- $\beta$ 2, TGF- $\beta$ 3, epidermal growth  
25 factor (EGF), platelet-derived growth factor (PDGF),  
fibroblast growth factor (FGF), insulin-like growth  
factor-I (IGF-I), and IGF-II.

          IGF-I and IGF-II are related in amino acid  
sequence and structure, with each polypeptide having a  
30 molecular weight of approximately 7500 daltons. IGF-I  
mediates the major effects of growth hormone and thus is  
the primary mediator of skeletal growth after birth.  
IGF-I has also been implicated in the actions of various  
other growth factors, since treatment of cells with such  
35 growth factors leads to increased production of IGF-I.

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Both IGF-I and IGF-II have insulin-like activity (hence the name) and are mitogenic (stimulating cell division) for various types of cells involved in the growth and differentiation of skeletal tissues such as muscle and bone, as well as non-skeletal tissues.

IGF can be measured in blood serum to diagnose abnormal growth-related conditions, e.g., gigantism, acromegaly, dwarfism, various growth hormone deficiencies, etc. Although IGF is produced in many tissues, most circulating IGF is believed to be synthesized in the liver.

Unlike most growth factors, the IGFs are present in substantial quantity in the circulation, but only a very small fraction of this IGF is free in the circulation or in other body fluids. Most IGF is complexed with IGF-binding proteins. IGF in the blood is mainly complexed with IGFBP-3, the major circulating IGF-binding protein. Almost all IGF circulates in a non-covalently associated ternary complex composed of IGF-I or -II, an IGF specific binding protein termed IGFBP-3, and a larger protein termed the Acid Labile Subunit (ALS). This ternary complex is composed of equimolar amounts of each of the three components. The ALS has no direct IGF binding activity and appears to bind only a pre-formed IGF/IGFBP-3 complex. The ternary complex of IGF + IGFBP-3 + ALS has a molecular weight of approximately 150,000 daltons. This ternary complex likely functions in the circulation "as a reservoir and a buffer for IGF-I and IGF-II preventing rapid changes of free IGF." Blum, W.F., et al., "Plasma IGFBP-3 Levels as Clinical Indicators", In Modern Concepts in Insulin-Like Growth Factors, E. M. Spencer, ed., Elsevier, New York, pages 381-393, 1991.

Having most circulating IGF in complexes is beneficial. Excess free IGF can cause serious



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hypoglycemia because IGF has insulin-like effects on circulating glucose levels. In contrast to the low levels of free IGFs and IGFBP-3, there is a substantial pool of free ALS in plasma which assures that IGF/IGFBP-3 complex entering the circulation immediately forms a ternary complex.

#### IGF Binding Proteins

IGFBP-3 is the most abundant IGF binding protein in the circulation. Recently, Wood et al. (Mol. Endocrin. (1988), 2:1176-85) and Spratt et al. (Growth Factors (1990), 3:63-72) described the cloning and expression of human IGFBP-3, whose structure is incorporated herein by reference. The gene for IGFBP-3 codes for 291 amino acids, the first 27 of which represent a characteristic signal sequence. Thus, the mature protein comprises 264 amino acids and has a predicted molecular weight of 28,749 (without glycosylation or other post-translational changes). When the human IGFBP-3 gene was expressed in Chinese hamster ovary ("CHO") cells and the conditioned culture medium was subjected to SDS electrophoresis and transferred to nitrocellulose membrane for ligand binding analysis, Spratt et al. reported "the presence of a 43-45 kd doublet, a 28 kd band and a minor 31 kd band" protein bands (p. 69), indicating there were post-translational changes. A side-by-side comparison revealed that the 43-45 kd doublet was also present in human serum.

Figures 12-15 show coding sequences and deduced amino acid sequences of IGFBP-3 suitable for various forms of use in the present invention.

It is unclear which tissue is the primary source of circulating IGFBP-3, although synthesis has been demonstrated in numerous cell types, including human fibroblasts, liver cells (most likely Kupfer cells) and

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osteoblasts. cDNA libraries that include the IGFBP-3 cDNA have been obtained from liver and other tissues. Vascular endothelial cells produce IGFBP-3 and may be the major source for systemic IGFBP-3.

5 IGFBP-3 has been purified from natural sources and produced by recombinant means. For instance, IGFBP-3 can be purified from natural sources using a process such as that shown in Martin and Baxter (J. Biol. Chem. (1986) 261:8754-60) IGFBP-3 also can be synthesized by  
10 recombinant organisms as discussed in Sommer, A. et al., In Modern Concepts of Insulin-Like Growth Factors, E. M. Spencer, ed., Elsevier, New York, pp. 715-728, 1991. This recombinant IGFBP-3 binds IGF-I with a 1:1 molar stoichiometry.

15 At least five other distinct IGF binding proteins have been identified in various tissues and body fluids. Although all these proteins bind IGFs, they each originate from separate genes and they have distinct amino acid sequences. Thus, the binding proteins are not  
20 merely analogs of a common precursor. For example, Spratt et al. compared the amino acid sequences of IGFBP-1, -2 and -3. Of the total 264 amino acids in the mature protein, only 28% of the amino acids are identical between IGFBP-3 and IGFBP-1, and 33% are identical  
25 between IGFBP-3 and IGFBP-2. Spratt et al. suggested that the similar portions of the binding proteins are the region(s) that bind IGF. Unlike IGFBP-3, the other IGFBPs in the circulation are not saturated with IGFs. All six known IGFBPs are reviewed and compared by  
30 Shimasaki and Ling, Prog. Growth Factor Res. (1991) 3:243-66.

Spencer et al. (1991) Bone 12:21-26; and Tobias et al. (1992) Endocrinology 131:2387-2392 report the stimulation of bone formation by IGF-I.

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The trabecular bone of rats, like that of humans, shows a coupling of bone formation to bone resorption, such that the increased resorption that occurs with estrogen deficiency entrains increased bone formation, which can be suppressed by inhibition of bone resorption. It has been established that in adult humans this coupling of formation and resorption involves a site specific sequence of events, in which bone resorption is normally followed, at the same site, by bone formation.

10 Frost (1985) Clin. Orthop. Rel. Res. 200:198-225.

There is also some evidence that bone formation can occur without previous bone resorption, primarily in those situations where demands for mechanical support of the skeleton are increased (modeling). Parfitt, A.M., et al. (1984) Calcif. Tissue Int. 36:5123-5128.

20 The present invention offers in vivo single or combination therapy for stimulating new bone formation through the administration of the IGF/IGFBP complex or through the administration of the IGF/IGFBP complex and an agent which inhibits bone resorption. These combinations provide more effective therapy for prevention of bone loss and replacement of bone.

#### Disclosure of the Invention

25 The present invention discloses the use of IGF and its binding protein for the initiation and promotion of bone formation and the regulation of bone remodeling. The IGF/IGFBP-3 complex can be used alone or in conjunction with an inhibitor of bone resorption.

30 The present invention discloses a method for stimulating bone formation in a subject who has a bone marrow disorder causing bone loss. The method comprises administering to the subject pharmaceutically effective doses of IGF-I and IGFBP-3, with or without an inhibitor  
35 of bone resorption.

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In another embodiment, the present invention discloses a method for stimulating bone formation in a subject who has a connective tissue disorder causing bone loss. The method comprises administering to the subject  
5 pharmaceutically effective doses of the IGF-I/IGFBP-3 complex.

In another embodiment, the present invention discloses a method for treating a subject who has drug-related osteoporosis. The method comprises administering  
10 to the subject pharmaceutically effective doses of the IGF-I/IGFBP-3 complex.

In another embodiment, the present invention discloses a method of stimulating bone formation in a subject who has bone loss due to pregnancy, lactation,  
15 chronic hypophosphatemia, hyperphosphatasia, insulin-dependent diabetes mellitus, anorexia nervosa, cadmium poisoning, juvenile osteoporosis. The method comprises administering to the subject pharmaceutically effective doses of the IGF-I/IGFBP-3 complex.

In another embodiment, the present invention discloses a method for stimulating bone formation in a subject who has periodontal bone loss. The method  
20 comprises administering to the subject pharmaceutically effective doses of IGF-I and IGFBP-3.

In another embodiment, the present invention discloses a method for stimulating bone formation in a subject who has bone loss associated with osteoarthritis,  
25 disuse, or prolonged exposure to reduced gravitational field. The method comprises administering to the subject pharmaceutically effective doses of IGF-I and IGFBP-3.  
30

In another embodiment, the present invention discloses a composition for inducing bone formation in a subject. The composition comprises IGF-I, IGFBP-3 and an inhibitor of bone resorption in a pharmaceutically  
35 acceptable excipient.

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Brief Description of the Figures

Figure 1 shows a schematic of the experimental design used in experiments carried out in support of the invention (Example 2);

5        Figures 2A and 2B show bar graphs of mean body weights (2A) and lean body mass (2B) of ovariectomized (OVX) animals treated for 8 weeks with rhIGF-I or rhIGF1/IGFBP-3 complex at the doses of IGF-I indicated, compared to no treatment (solid bars) and sham operated controls  
10      (solid line);

      Figure 3 shows a bar graph of endochondral bone growth) of ovariectomized (OVX) animals treated for 8 weeks with rhIGF-I or rhIGF-I/IGFBP-3 complex at the doses of IGF-I indicated, compared to no treatment (solid  
15      bars) and sham operated controls (solid line);

      Figures 4A and 4B show bar graphs of tibial periosteal bone formation rate (4A) and tibial endocortical resorption (4B) of ovariectomized (OVX) animals treated for 8 weeks with rhIGF-I or  
20      rhIGF-I/IGFBP-3 complex at the doses of IGF-I indicated, compared to no treatment (solid bars) and sham operated controls (solid line);

      Figures 5A-5CC show matching UV micrographs (5A-5C, 3X magnification; 5CC, 12X magnification of  
25      periosteal envelope region shown in 5C) of ground 40  $\mu$ m thick tibial cross-sections from sham (5A), OVX (5B), and OVX rats treated with 7.5 mg/kg rhIGF-I complexed with rhIGFBP-3 (5C, 5CC) where new bone formation on the periosteal envelope is indicated by the arrows;

30      Figures 6A-6D show polarized light micrographs (12.5 X magnification) of unstained, 40  $\mu$ m thick cross sections from Sham operated animals (6A), OVX animals (6B), and from animals treated with 7.5 mg/kg rhIGF-I complexed with rhIGFBP-3 (6C, 6D), where outer lamellar  
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layers are indicated by arrowheads and inner lamellar layers are indicated by arrows;

Figures 7A-7D show bar graphs of bone formation rates of cancellous bone in the metaphyses (7A),  
5 epiphyses (7B), lumbar vertebral bodies (7C) and femoral neck region (7D), of ovariectomized (OVX) animals treated for 8 weeks with rhIGF-I or rhIGF-I/IGFBP-3 complex with the doses of IGF-I indicated, compared to no treatment (solid bars) and sham operated controls (solid line),  
10 where an asterisk indicates  $p < 0.05$  compared to OVX group;

Figures 8A-8C show UV micrographs of cancellous trabeculae in distal femoral epiphysis from sham-operated control (8A), OVX (8B) and 7.5 ml/kg rhIGF-I/IGFBP-3  
15 complex (8C) treated rats, where arrows indicate resorption pits and arrowheads indicate newly formed bone;

Figures 9A-9C show matching micrographs (3X magnification) of 4  $\mu$ m section taken from distal femurs stained by the mineral-staining method of von Kossa from  
20 sham-operated controls (9A) OVX (9B) and 7.5 mg/kg rhIGF-I/IGFBP-3 complex treated rats (9C);

Figures 10A-10C show cross-sectional images (2.5X magnification) of sections taken from femoral mid-neck region of sham-operated control (10A), OVX rats  
25 (10B) and from rats treated with 7.5 mg/kg rhIGF-I complexed with IGFBP-3 (10C);

Figure 11 shows the amino acid sequences of human IGF-I used in the present invention (SEQ ID NO:1);

30 Figure 12 shows the amino acid sequence of human IGFBP-3 (SEQ ID NO:2), where an asterisk at position 5 indicates substitution of glycine for alanine according to naturally occurring heterogeneity in the sequence);

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Figure 13 shows a nucleotide sequence of human IGFBP-3 (SEQ ID NO:3 and SEQ ID NO:4) used to produce rhIGFBP-3 for use in accordance with the invention;

Figure 14 shows an alternate nucleotide sequence (SEQ ID NO:5) for production of rhIGFBP-3 for use in accordance with the invention; and

Figure 15 shows a second alternate nucleotide sequence (SEQ ID NO:6 and SEQ ID NO:7) for production of rhIGFBP-3 for use in accordance with the invention.

### Modes for Carrying Out the Invention

#### A. Definitions

It must be noted that as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise.

The term "connective tissue disorder" includes conditions such as osteogenesis imperfecta, Ehlers-Danlos syndrome, Marfans syndrome, cutis laxa, homocystinuria, Mankes's syndrome and scurvy. These have been reported to be associated with and cause bone loss.

"Drug-related osteoporosis" means osteoporosis whose only identified cause is a drug. The definition includes all known drugs which cause osteoporosis, as well as those drugs later discovered to cause osteoporosis. Examples of drugs known to cause osteoporosis include corticosteroids, heparin, oral anticoagulants, anticonvulsants, methotrexate, thyroid hormone, lithium and gonadotrophin-releasing analogs.

"Insulin-like growth factor (IGF)" comprises a family of factors, including but not limited to IGF-I and IGF-II. IGF is a polypeptide having a molecular weight of about 7500 daltons. IGF can be obtained from natural sources or prepared by recombinant means. Preferably IGF is IGF-I from human sources. Most preferably, IGF is

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is IGF-I from human sources. Most preferably, IGF is human IGF-I made by recombinant means, such as by the methods detailed in Example 3 herein, and designated rhIGF-I.

5 "Insulin-like growth factor binding protein (IGFBP)" comprises a family of binding proteins, including but not limited to IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 and IGFBP-6. Each IGFBP can be obtained from natural sources or prepared by recombinant means,  
10 such as detailed in Example 4 herein. At least one form of IGFBP (for example, IGFBP-3) complexes with IGF and with a third molecule known as ALS. IGFBP also can be a mixture of any combination of the six IGFBP's. Such a mixture would take advantage of the different binding  
15 affinities for IGF-I and/or IGF-II, the ability of some IGFBP's to bind to cell surfaces, and the different half-lives.

A "therapeutic composition" as used herein is defined as comprising the IGF binding protein (IGFBP)  
20 with IGF and a bone resorption inhibitor. The therapeutic composition can also contain excipients such as water, minerals and carriers such as protein.

The method of the present invention contemplates treating a subject in need of local bone  
25 repair or replacement with IGFBP in combination with IGF with or without a bone resorption inhibitor. The IGF may be any of the IGF family, including but not limited to, IGF-I and IGF-II, or a combination thereof. When IGF-I and IGF-II are combined, the ratio of IGF-I to IGF-II  
30 ranges from 0.01:1 to 99:1.

"IGFBP-1" is an IGF-binding protein whose molecular structure was disclosed by Brewer et al., Biochem. Biophys. Res. Comm. (1988) 152(3):1289-1297, and by Drop et al. in PCT Publication No. WO 89/98667,  
35 published on September 21, 1989, and is incorporated



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herein by reference. Human IGFBP-1 has 234 amino acids and a molecular weight of about 28 kd.

"IGFBP-2" comprises 289 amino acids (human) and has a molecular weight of 36 kd under nonreducing conditions. The amino acid sequence of human IGFBP-2 was determined from cDNA clones isolated from a human fetal liver library by Binkert et al. EMBO J. (1989) 8:2493-2502, and is incorporated herein by reference. IGFBP-2 also may bind to cell surfaces. IGFBP-2 has a preference for IGF-II, and thus is preferred in formulations comprising IGF-II.

"IGFBP-3" is the preferred IGFBP in the IGF/IGFBP complex. Native and recombinant IGFBP-3, as well as some N-terminal and C-terminal fragments, bind IGF-I and IGF-II. Human IGFBP-3 comprises 264 amino acids and has three potential N-linked glycosylation sites. IGFBP-3 is the major IGFBP in blood.

Nearly all IGF-I or IGF-II in blood is bound to IGFBP-3. The IGF/IGFBP-3 complex normally circulates in the form of a complex in humans and other mammals and avians. This complex associates with a third protein (ALS), which is present in excess over the concentration of IGF and IGFBP-3. Therefore, in the circulation, ALS is found both associated with the IGF/IGFBP-3 complex and in free form. The resultant ternary complex has a size of about 150 kD. Administration of a pre-formed complex of IGF and IGFBP-3, either from natural or recombinant sources, results in the formation of the ternary complex with the normally excess ALS. This type of treatment appears to produce a long term increase in the level of circulating IGF, which is gradually released from the ternary complex. This mode of administration avoids the detrimental side effects associated with administration of free IGF-I, e.g., hypoglycemia, suppression of growth hormone and ALS production, and release of endogenous

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IGF-II since administered exogenous free IGF-I replaces endogenous IGF-II in normally circulating IGF-II/IGFBP-3 complexes.

"IGFBP-4" and "IGFBP-6" are glycosylated proteins which are widely distributed in the body. The primary structure of IGFBP-4 was reported by Shimasaki et al. Mol. Endocrinol. (1990) 4:1451-1458, and is incorporated herein by reference. IGFBP-6, whose cDNA has been isolated by Shimasaki et al. (Mol. Endocrinol. (1991) 5:938-48), has a much greater affinity for IGF-II than for IGF-I and may be preferred with formulations containing IGF-II. This reference is incorporated herein by reference.

"IGFBP-5" is a 252-amino acid protein which is not glycosylated. Shimasaki et al. (J. Biol. Chem. (1991) 266:10646-53) cloned human IGFBP-5 cDNA from a human placenta library, and is incorporated herein by reference.

Depending on the binding, metabolic and pharmacokinetic characteristics required in the IGF/IGFBP complex formulation, these binding proteins can be added to the complex formulation in various proportions. These IGFBP's can be combined in a wide variety of ratios with IGF-I and/or IGF-II.

The term "inhibition of bone resorption" refers to prevention of bone loss, especially the inhibition of removal of existing bone either from the mineral phase and/or the organic matrix phase, through direct or indirect alteration of osteoclast formation or metabolism. Thus, the term "inhibitor of bone resorption" as used herein refers to agents that prevent bone loss by the direct or indirect alteration of osteoclast formation or metabolism.

The term "bone remodeling" refers to that process which renews the skeleton and repairs microdamage

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before it accumulates to the point of loss of skeletal integrity. Most adult human metabolic bone disease results from derangement of remodeling processes. The individual remodeling packets are known as "basic multicellular units" (BMU). Remodeling which occurs in BMUs follows a preprogrammed sequence: activation → resorption → formation (ARF). (Recker, R.R., "Bone Histomorphometry: Techniques and Interpretation." Boca Raton, FL, 1983, pp. 37-57.)

"Bone formation" includes increases in the number and/or activity of BMU (Basic Multicellular Units) producing new bone. This process can take place anywhere in the skeleton and affect cancellous and/or cortical bone with no regard to functional differences and turn-over rates which particular bone sites might have in the skeleton. (Frost (1986) Intermediary Organization of the Skeleton, Vols. I and II. CRC Press, Boca Raton, FL.)

The term "osteogenically effective" means that amount which affects the formation and differentiation of mature bone or early bone progenitor cells. As used herein, an osteogenically effective dose is also "pharmaceutically effective."

The term "subject" as used herein refers to a living vertebrate animal such as a mammal or bird in need of treatment, i.e., in need of bone repair or replacement. Such need arises in connective tissue disorders such as osteogenesis imperfecta, Ehlers-Danlos syndrome, Marfans syndrome, cutis laxa, homocystinuria, Menkes's syndrome and scurvy; osteoporosis related to such drugs as corticosteroids, heparin, oral anticoagulants, anticonvulsants, methotrexate, thyroid hormone, lithium and gonadotrophin-releasing analogs; pregnancy; lactation; chronic hypophosphatemia; hyperphosphatasia; insulin-dependent diabetes mellitus; anorexia nervosa; Paget's disease of bone; cadmium

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poisoning; periodontal disease; osteoarthritis, disuse of the musculoskeletal system such as occurs during prolonged bed rest, paralysis or semi-paralysis; and prolonged exposure to reduced gravitational field.

5           The term "treatment" as used herein shall mean (1) providing a subject with an amount of a substance sufficient to act prophylactically to prevent the development of bone loss; or (2) providing a subject with a sufficient amount of a substance so as to alleviate or  
10       eliminate the problems associated with the bone loss.

B.           General methods

Drugs which prevent bone loss and/or add back lost bone have been evaluated in the ovariectomized rat.  
15       This animal model is well established in the art (see, for example, Wronski et al. (1985) Calcif. Tissue Int. 37:324-328; Kimmel et al. (1990) Calcif. Tissue Int. 46:101-110; Durbridge et al. (1990) Calcif. Tissue Int. 47:383-387; and Miller et al. (1991) Bone 12:439-446;  
20       these references are hereby incorporated in their entirety). Wronski et al. ((1985) Calcif. Tissue Int. 43:179-183)) describe the association of bone loss and bone remodeling in the ovariectomized rat.

Examples of inhibitors of bone resorption  
25       include estrogen, such as conjugated estrogen, tamoxifen, bisphosphonates, calcitonins, or other small peptides or molecules that may inhibit bone resorption. (Turner et al. (1987) J. Bone Mineral Res. 2:115-122; Wronski et al. (1988) Endocrinology 128:681-686; and Wronski et al.  
30       (1989) Endocrinology 125:810-816; Pfeilshifter et al. (1987) Proc. Natl. Acad. Sci. U.S.A. 84:2024-2028; Turner et al. (1988) Endocrinology 122:1146-1150). An example of a small peptide is echistatin, which includes the arginine-glycine-aspartate (RGD) sequence which is  
35       recognized by some cell surface adhesion receptors and

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apparently disrupts osteoclast interaction (Fisher et al. (1993) Endocrinology 132: 1411-13). Another example of a bone resorption factor is OPF or osteoclastopoietic factor (PCT Publication WO 93/01827 published 4 February 1993).

5 Other agents that are under investigation or have been proposed to inhibit bone resorption include mithramycin, gallium nitrate, glucocorticoids, transforming growth factor- $\beta$  (TGF- $\beta$ ), interferon- $\gamma$  and amylin. (Zaidi et al. (October 1992) Curr. Opin. Therapeutic Patents, pp. 1517-10 38).

The entire molecule of a particular inhibitor may be used, or alternatively, only a functional part of the inhibitor molecule may be used. Bisphosphonates include, but are not limited to, pamidronate acid, 15 alendronate, tiludronate, risedronate and other experimental compounds.

Certain growth factors can inhibit bone resorption, or are anti-resorptive. Examples of such growth factors include but are not limited to the 20 transforming growth factors- $\beta$ .

In accordance with the method of the present invention, the formulation comprises a complex of IGF and IGFBP administered with or without a bone resorption inhibitor.

25 Preferably, the IGF is IGF-I, although IGF-II can be useful. Preferably, the IGFBP is IGFBP-3. Because IGF and IGFBP-3 naturally bind in a 1:1 molar ratio, a composition of equimolar amounts of IGF and IGFBP-3 is preferred. Nevertheless, the composition can 30 be formulated with IGF:IGFBP-3 molar ratios ranging from 0.5:1 to 1.5:1. More preferably, the molar ratio is 0.9:1 to 1.3:1; and most preferably, the composition is formulated with approximately a 1:1 molar ratio.

In accordance with the method of the present 35 invention, IGF and IGFBP-3 are human proteins obtained

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from natural or recombinant sources. Most preferably, IGF and IGFBP-3 are human IGF-I and IGFBP-3 made by recombinant means and designated rhIGF-I and rhIGFBP-3, respectively. rhIGFBP-3 can be in glycosylated or non-glycosylated form. *E. coli* is a source of the non-glycosylated IGFBP-3. Glycosylated IGFBP-3 can be obtained from CHO-cells.

The method of the present invention provides for formulating the complex in modes which are readily apparent to those skilled in the art. Preferably, the IGF and IGFBP-3 are complexed prior to administration to the treated individual. Preferably, the complex is formed by mixing approximately equimolar amounts of IGF-I and IGFBP-3 dissolved in physiologically compatible carriers such as normal saline solution or phosphate buffered saline solution. Most preferably, a concentrated solution of rhIGF-I and a concentrated solution of IGFBP-3 are mixed together for a sufficient time to form an equimolar complex.

Pharmaceutical compositions of the invention which include IGF, IGFBP and an inhibitor of bone resorption for administration include osteogenically effective amounts of IGF and IGFBP to promote bone formation and an inhibitory amount of a bone resorption inhibitor, in addition to a pharmaceutically acceptable excipient. Suitable excipients include most carriers approved for parenteral administration, including water, saline, Ringer's solution, Hank's solution, and solutions of glucose, lactose, dextrose, ethanol, glycerol, albumin, plasma, other protein-containing solutions and the like. These compositions may optionally include stabilizers, antioxidants, antimicrobials, preservatives, buffering agents, surfactants, and other accessory additives. The IGF/IGFBP complex and inhibitor of bone

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resorption may also be delivered in a slow release form from a suitable carrier.

Various vehicles may be used with the present invention. A thorough discussion of suitable vehicles for parenteral administration may be found in E.W. Martin, "Remington's Pharmaceutical Sciences" (Mack Pub. Co., current edition). Sections relating to the excipient vehicles and formulating are incorporated herein by reference. Such formulations are generally known to those skilled in the art and are administered systemically to provide systemic treatment.

The IGF/IGFBP complex and an agent which inhibits bone resorption may be administered simultaneously, as a single composition to the subject, or sequentially. If administered sequentially, the period between the administration of the IGF/IGFBP complex and the inhibitor of bone resorption is typically one day to one year, and preferably, one week to six months. If the IGF/IGFBP complex and the agent which inhibits bone resorption are administered as a single composition, the molar ratio of IGF/IGFBP complex to inhibitor of bone resorption varies considerably depending on the type of bone resorption inhibitor and the formulation of the IGF/IGFBP complex. The ratio for most compounds is between about 100:1 to 1:100. Furthermore, if administered as a single composition, the IGF/IGFBP complex and the inhibitor of bone resorption may be administered as separate molecules in the composition, or the respective molecules may be conjugated or fused according to techniques well known in the art.

The precise dosage necessarily varies with the age, size, sex and condition of the subject, the nature and severity of the disorder to be treated, and the like. Thus, a precise effective amount cannot be specified in

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advance and must be determined by the care giver. However, appropriate amounts have been and can be determined by routine experimentation with animal models, as described below.

5                   In general terms, an effective dose of IGF/IGFBP complex for systemic treatment will range from about 1  $\mu$ g to about 10 mg IGF/kg of body weight. An effective dose for an inhibitor of bone resorption depends upon the particular inhibitor selected for  
10 administration. For example, an effective dose of estrogen is about 0.25 to 1.5 mg/day. An effective dose for bisphosphonates varies but is generally between about 0.05  $\mu$ g/kg to about 15 mg/kg of body weight. An effective dose for calcitonin is about 0.05 IU  
15 (International Units or Medical Research Council Units)/kg to about 2.5 IU/kg of body weight.

Effective doses for local administration range from about 0.01  $\mu$ g to 1 mg of IGF/IGFBP complex.

The methods and compositions of the invention  
20 are useful for treating bone fractures, defects, and disorders which result in weakened bones. Among these disorders are bone marrow dyscrasias such as plasma cell dyscrasias, leukemia, lymphomas, systemic mastocytosis, anemias, lipidoses and mucopolysaccharidoses; connective  
25 tissue disorders such as osteogenesis imperfecta, Ehlers-Danlos syndrome, Marfans syndrome, cutis laxa, homocystinuria, Menkes's syndrome and scurvy; osteoporosis related to such drugs as corticosteroids, heparin, oral anticoagulants, anticonvulsants,  
30 methotrexate, thyroid hormone, lithium and gonadotrophin-releasing analogs; pregnancy; lactation; chronic hypophosphatemia; hyperphosphatasia; insulin-dependent diabetes mellitus; anorexia nervosa; Paget's disease of bone; juvenile osteoporosis; cadmium poisoning;  
35 periodontal disease; and osteoarthritis. In addition,



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methods and compositions of the present invention are useful in treating weakened bones resulting from disuse of the musculoskeletal system, such as occurs as a consequence of prolonged bed rest or exposure to decreased gravitational fields.

In accordance with one method of use, the IGF/IGFBP complex may be administered locally to a specific area in need of bone growth or repair, with either the concomitant administration of the inhibitor of bone resorption at the site, or the administration of the inhibitor of bone resorption in a separate vehicle. Thus, the IGF/IGFBP complex and/or inhibitor of bone resorption may be implanted directly at the site to be treated, for example, by injection or surgical implantation in a sustained-release carrier. Suitable carriers include hydrogels, controlled- or sustained-release devices (e.g., an Alzet® minipump), polylactic acid, and collagen matrices. Presently preferred carriers are formulations of atelopeptide collagen containing particulate calcium phosphate mineral components, such as combinations of homologous or xenographic fibrillar atelopeptide collagen (for example Zyderm® Collagen Implant, available from Collagen Corporation, Palo Alto, California) with hydroxyapatite-tricalcium phosphate (HA-TCP, available from Zimmer, Inc., Warsaw, IN). It is presently preferred to administer implant compositions containing an IGF/IGFBP complex and/or an inhibitor of bone resorption in a collagen/mineral mixture implant.

IGF/IGFBP complex and/or an inhibitor of bone resorption, delivered in sustained-release vehicles is also particularly useful for improving implant fixation, for example, for improving ingrowth of new bone into a metal prosthesis in joint reconstruction and dental or orthopedic implants. Alternatively, the IGF/IGFBP

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complex may be delivered in the implant, with the inhibitor delivered in a separate vehicle, and vice versa.

Dental and orthopedic implants can be coated with the IGF/IGFBP complex in combination with an inhibitor of bone resorption to enhance attachment of the implant device to the bone. Alternatively, the IGF/IGFBP complex can be used to coat the implant, and the inhibitor of bone resorption can be administered concomitantly or sequentially in a separate vehicle, and vice versa.

In general, implant devices may be coated with the IGF/IGFBP complex and/or an inhibitor of bone resorption as follows. The IGF/IGFBP complex (and the inhibitor of bone resorption, if desired) is dissolved at a concentration in the range of 0.01 mg/ml to 200 mg/ml in phosphate-buffered saline (PBS) containing 2 mg/ml serum albumin. The porous end of an implant is dipped in the solution and is air dried (or lyophilized) or implanted immediately into the bony site. The viscosity of the coating solution is increased, if desired, by adding hyaluronate at a final concentration of 0.1 mg/ml to 100 mg/ml or by adding other pharmaceutically acceptable excipients. Alternatively, the solution containing the IGF/IGFBP complex (and the inhibitor of bone resorption, if desired) is mixed with collagen gel or human collagen (e.g. Zyderm® Collagen Implant, Collagen Corp., Palo Alto, CA) to a final collagen concentration of 2 mg/ml to 100 mg/ml to form a paste or gel, which is then used to coat the porous end of the implant device. The coated implant device is placed into the bony site immediately or is air dried and rehydrated with PBS prior to implanting, with the objective of maximizing new bone formation into the implant while

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minimizing the ingrowth of soft tissue into the implant site.

C. Examples

5           The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to extract, isolate, formulate and use the compositions and methods of the invention and are not intended to limit the scope  
10 of what the inventors regard as their invention. Efforts have been made to insure accuracy with respect to numbers used (e.g., amounts, times, temperature, etc.), but some experimental error and deviations should be accounted for. Unless indicated otherwise, parts are parts by  
15 weight, temperature is in degrees centigrade, pressure is at or near atmospheric, and other parameters are conventional and in accordance with those normally accepted by those skilled in the art.

20                           Example 1

          This example shows the use of free IGF-I and the IGF-I/IGFBP-3 complex in female rats with ovariectomy-induced osteoporosis. In this experiment, rats were treated with human recombinant IGF-I and  
25 IGFBP-3. The rhIGF-I (Ciba-Geigy) was synthesized in yeast and provided in sterile water and stored at -70°C. The rhIGFBP-3 was made according to the procedure described in Example 4 (Celtrix Pharmaceuticals, Inc., Santa Clara, CA) and was dissolved in phosphate-buffered  
30 saline and stored at -70°C until use. Prior to administration, these solutions were thawed, and sufficient amounts of IGF-I and IGFBP-3 were mixed to provide equimolar amounts of the two proteins. This experiment demonstrates the ability of the IGF-I/IGFBP-3  
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complex to increase trabecular bone mass and other parameters in ovariectomized rats.

In this example, young female rats of 90-100 g body weight were ovariectomized by the dorsal route and were divided into six groups of eight rats each. An additional group consisted of eight intact, age-matched sham operated control rats. Six weeks after ovariectomy, rats were treated with vehicle or with one of the following combinations of IGF-I and IGFBP-3 or IGF alone, as indicated. In these experiments, the amounts of IGF and IGFBP used were calculated to provide a 1:1 molar ratio of IGF:IGFBP:

- Group 1: Sham Operated Controls; Vehicle
- Group 2: Ovariectomized Controls; Vehicle
- Group 3: Ovariectomized; 2.5 mg/kg IGF-I complexed to IGFBP-3 (9.5 mg/kg)
- Group 4: Ovariectomized; 0.25 mg/kg IGF-I complexed to IGFBP-3 (0.95 mg/kg)
- Group 5: Ovariectomized; 0.025 mg/kg IGF-I complexed to 0.095 mg/kg IGFBP-3
- Group 6: Ovariectomized; 2.5 mg/kg IGF-I
- Group 7: Ovariectomized; 0.25 mg/kg IGF-I

The complex was formed by mixing equimolar amounts of IGFBP-3 (dissolved in phosphate buffered saline (PBS), pH 6.0) and IGF-I (dissolved in 10 mM sodium acetate, pH 5.5) in the minimum volume feasible, and incubating the mixture overnight at 4°C. The complex was then diluted with PBS, pH 6.0, containing 0.1% rat serum albumin. The solutions were divided into aliquots containing the amount of material needed for one day, and stored at -70°C until needed. The controls received the dilution buffer.

The rats were treated for 22 days. The test substances were administered six times per week by one

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daily subcutaneous injection. One day before treatment was started and on the 17th day of treatment, 20 mg/kg of calcein was given by intraperitoneal injection. Calcein and tetracycline are markers for bone mineralization and are used to estimate the amount of bone formation between administrations. Similarly, on the tenth day 20 mg/kg of demeclocycline was administered. On day 23, 24 hours after the last injection, the rats were killed by anesthesia with carbon dioxide.

Body weights were recorded throughout the experiment. At autopsy, 0.1 ml of blood was taken for the determination of blood glucose. Serum was prepared from the rest of the blood, and total serum IGF-I levels were determined by radioimmunoassay (RIA). Gastrocnemius muscle, periuterine fat and uterus were removed, dissected free of connective tissue, and weighed.

The amount of trabecular and cortical bone was determined according to Gunness-Hey ((1984) Metab. Bone Dis. & Rel. Res. 5:177-81), incorporated herein by reference. Briefly, the femurs were cut in half at the mid-diaphysis using a dental saw. The proximal halves were discarded. The epiphysis of the distal half was cut off using a scalpel, and the bone was split into sagittal halves. The marrow was flushed out with water. With a dental curette, the metaphyseal trabecular bone was scraped out of both cortical shells, combined and put into 5% trichloroacetic acid (TCA). The two pieces of the remaining cortical bone were also combined and put into a separate tube with 5% TCA. After this preparation stood 16 hours at room temperature, the TCA extract was used for the determination of calcium by atomic absorption spectroscopy. The remaining demineralized matrix was washed successively with ethanol and methylene chloride, and dried under vacuum. After determination of the dry weight, the matrix was hydrolyzed with 6 M HCl at

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120°C for 5 hours. In the hydrolysate hydroxyproline was determined by a standard colorimetric assay (Jamall et al. (1981) Analyt. Biochem. 112:70-75).

The results of this experiment are detailed in  
5 Tables 1 and 2 and summarized below.

When ovariectomized ("Ovx" in the tables) control rats were compared to sham operated control animals, no significant differences were observed in cortical bone weight or hydroxyproline (Table 2).  
10 However, cortical bone calcium was significantly increased in ovariectomized rats (7%). This increase may be due to increased longitudinal bone growth and increase in organ size. In the ovariectomized rats, trabecular bone weight, calcium and hydroxyproline were reduced by  
15 65% (Table 1).

In ovariectomized rats treated with IGF-I alone, both doses (2.5 mg/kg and 0.25 mg/kg) increased trabecular bone mass; however, the lower dose was more effective than the higher dose. The lower dose increased  
20 various parameters 79%-118%; whereas, the higher dose increased various parameters by 57%-67%. There were marginal changes in other parameters.

Ovariectomized rats treated with IGF-I/IGFBP-3 complex showed an increase in various trabecular bone  
25 parameters (Table 1). Significant increases in trabecular calcium, dry weight, and hydroxyproline were observed at an IGF-I/IGFBP-3 dose of 12 mg/kg (2.5 mg/kg IGF-I and 9.5 mg/kg IGFBP-3). Cortical bone matrix dry weight was also increased significantly by treatment with  
30 12 mg/kg IGF-I/IGFBP-3 complex (Table 2).

Table 1Treatment Groups

	Group 1 (Sham Operated)	Group 2 (Ovx Control)	Group 3	Group 4	Group 5	Group 6	Group 7
IGF-I (mg/kg)	-	-	2.5	0.25	0.025	2.5	0.25
IGFBP-3 (mg/kg)	-	-	9.5	0.95	0.095	-	-

Trabecular Bone<sup>1</sup>

Calcium (mg) (%)	4.0±0.4 (+186)*	1.4±0.1 -	2.7±0.2 (+93)	1.8±0.2 (+29)	1.6±0.1 (+14)	2.2±0.2 (+57)*	2.5±0.2 (+79)*
Dry Weight (mg) (%)	2.9±0.4 (+164)*	1.1±0.1 -	2.1±0.2 (+91)	1.7±0.2 (+55)	1.6±0.3 (+45)	1.8±0.1 (+64)*	2.4±0.2 (+118)*
Hydroxyproline (mg) (%)	269±35 (+186)*	94±11 -	221±20 (+135)*	122±20 (+30)	157±30 (+67)	157±30 (+67)	169±16 (+80)*

<sup>1</sup> % are compared to ovariectomized, vehicle treated control values (Group 2)  
 \* p < 0.05 compared to OvX control

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**Table 2**Treatment Groups

	Group 1 (Sham Operated)	Group 2 (Ovx Control)	Group 3	Group 4	Group 5	Group 6	Group 7
IGF-I (mg/kg)	-	-	2.5	0.25	0.025	2.5	0.25
IGFBP-3 (mg/kg)	-	-	9.5	0.95	0.095	-	-
<u>Cortical Bone<sup>1</sup></u>							
Calcium (mg) (%)	24.6±0.5 (-7)*	26.5±0.7 -	26.1±0.9 (-2)	26.7±0.7 (+1)	23.2±0.5 (-12)*	24.0±0.6 (-9)*	23.5±0.6 (-11)*
Dry Weight (mg) (%)	52.8±3.4 (-7)	56.8±2.7 -	64.7±2.7 (+14)*	56.8±3.2 (±0)	59.1±2.0 (+4)	6.81±3.6 (+20)*	55.1±0.2 (-3)
Hydroxyproline (mg) (%)	2679±177 (-8)	2925±143 -	3144±146 (+7)	2978±157 (+2)	3050±107 (+4)	3331±102 (+14)*	3141±194 (+7)

<sup>1</sup> % are compared to ovariectomized, vehicle treated control values (Group 2)  
 \* p < 0.05 compared to Ovx control



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Example 2

Thirteen week old female Sprague Dawley rats (Charles River Associates) were housed for approximately three weeks before undergoing either ovariectomy (OVX) or sham operation (Sham). All OVXs were successful as determined by uterine weights measured at sacrifice. Eight weeks after surgery (designated day 0) the rats underwent a Dual Energy X-ray Absorptometry (DEXA) spine scan for baseline measurements of bone mineral density of vertebrae L3 through L6 (BMD) and were assigned into groups (see below). Attempts were made to exclude the "outliers" and have similar mean spine BMD values and body weights among the various groups.

The rhIGF-I/IGFBP-3 was prepared by mixing equimolar quantities of rhIGF-I and rhIGFBP-3. The vehicle employed for both rhIGF-I and rhIGF-I/IGFBP-3 was phosphate buffered saline (PBS), pH. 6.0.

Treatments (by daily subcutaneous injection) began the day after the baseline DEXA spine measurements (day 1) and continued for 56 days. Two additional "pretreatment" groups of rats (OVX and Sham) were sacrificed on day 0 to provide baseline data.

The rats were divided into seven groups according to the experimental paradigm illustrated in Fig. 1:

- (1) Sham rats treated with saline ("Sham control"; n=9).
- (2) OVX rats treated with saline ("OVX control"; n=11).
- (3) OVX rats treated with 0.9 mg/kg/day of rhIGF-I (n=7).
- (4) OVX rats treated with 2.6 mg/kg/day of rhIGF-I (n=7).
- (5) OVX rats treated with 0.9 mg/kg/day of rhIGF-I in equimolar ratio with IGFBP-3 (n=8).

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(6) OVX rats treated with 2.6 mg/kg/day of rhIGF-I in equimolar ratio with IGFBP-3 (n=8).

(7) OVX rats treated with 7.5 mg/kg/day of rhIGF-I in equimolar ratio with IGFBP-3 (n=8).

5 a. Serum Measurements

On day 22 food was withdrawn from the rats at about 7 a.m. and 150  $\mu$ l of blood was obtained from the tail of each rat under isoflurane anesthesia between 10 a.m. and noon. Immediately after the blood was  
10 collected, each rat received its normal daily treatment injection, and a second blood sample was obtained exactly two hours after the injection. Food was returned to the rats only after this second bleeding. Plasma glucose levels were measured by a standard colorimetric assay  
15 involving the oxidation of O-dianisidine by the peroxide produced from glucose as a result of treatment with glucose oxidase according to standard procedures well known in the art. (Table 3)

During the seventh week of the study, blood was  
20 again obtained from the tail during the normal DEXA scan. These samples were analyzed for glycosylated hemoglobin levels using an affinity chromatographic procedure, specifically the "GLYCO-TEK" assay kit (Helena Laboratories, Beaumont, TX) with the eluted hemoglobins  
25 detected by their absorbances at 405 nm.

Serum obtained at sacrifice was analyzed for levels of total IGF-I, rhIGF-I and rhIGFBP-3. Serum IGF-I levels were determined by two separate assays. Each assay was performed once on the entire set of  
30 samples.

First, a Nichols Institute RIA procedure was employed to quantitate the combined concentrations of endogenous rat IGF-I and the injected rhIGF-I. Samples of 80  $\mu$ l were extracted with 900  $\mu$ l of 87.5% 2N HCl/12.5%  
35 ethanol, centrifuged, and then 200  $\mu$ l of the supernatant

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was neutralized with 100  $\mu$ l of 855 mM Tris buffer pH 11.0. This neutralized extract was kept at -20°C for one hour, centrifuged, and then diluted 31-fold with phosphate buffered saline (PBS) prior to analyzing 50  $\mu$ l by RIA. A preliminary assay indicated that serum obtained from hypophysectomized rats (n=6) gave values below the limit of detection (66 ng/ml) and the assay gave a linear response when the volume of serum was varied from 10 to 100  $\mu$ l.

The second assay quantitated the serum levels of rhIGF-I by immunoradiometric assay (IRMA), according to protocols provided by the manufacturer, with a sample size of 10  $\mu$ l. A pooled rat serum sample containing 555 ng/ml IGF-I by the Nichols Institute RIA procedure gave values below the limit of detection (50 ng/ml) of this assay. In addition, serum levels of rhIGFBP-3 on 10  $\mu$ l samples were determined by RIA. The assay kits and procedures for both rhIGF-I and rhIGFBP-3 were obtained from Diagnostic Systems Laboratories (Webster, TX).

Serum IGF-I and IGFBP-3 levels and blood glycosylated hemoglobin values are shown in Table 4.

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**Table 3**Fasting Plasma Glucose (mg/dl)  
(Day 22 of Study)

Group	Treatment	Time = 0 hours		Time = 2 hours		Student's P Value
		Glucose	SEM	Glucose	SEM	
1	Sham	191	6	162	6	0.003
2	OVX-Saline	183	8	197	4	0.120
3	0.9 mg/kg IGF-I	179	8	146	24	0.223
4	2.6 mg/kg IGF-I	163	6	73	11	0.001
5	0.9 mg/kg IGF-I + IGFBP-3	175	7	195	9	0.093
6	2.6 mg/kg IGF-I + IGFBP-3	192	6	204	10	0.349
7	7.5 mg/kg IGF-I + IGFBP-3	174	6	161	14	0.432

Table 4

Serum IGF-I and IGFBP-3 Levels  
and Blood Glycosylated Hemoglobin Values

Group	Treatment	Total IGF-I (ng/ml)	rhIGF-I (ng/ml)	rhIGFBP-3 (ng/ml)	Glycosylated Hemoglobin (%)
1	Sham-operated + Saline	not measured	not measured	not measured	6.7±0.3 (9)
2	OVX-Saline	487±33 (9)	<50 (8)	<250 (8)	7.2±0.2 (11)
3	0.9 mg/kg rhIGF-I	619±73 (5)	204±56 (5)	<250 (5)	7.3±0.3 (7)
4	2.6 mg/kg rhIGF-I	633±75 (7)	329±61 (7)	<250 (7)	7.3±0.3 (7)
5	0.9 mg/kg rhIGF-I/ IGFBP-3	1026±162 (6)	1141±294 (6)	2441±775 (6)	7.3±0.4 (8)
6	2.6 mg/kg rhIGF-I/ IGFBP-3	988±63 (7)	1391±10 (7)	2372±525 (7)	7.3±0.4 (8)
7	7.5 mg/kg rhIGF-I/ IGFBP-3	1783±273 (8)	2103±123 (8)	8217±2247 (8)	7.3±0.4 (8)
ANOVA P Value (Dose)		<0.001	-	-	0.83
ANOVA P Value (Treatment)		0.014	-	-	0.69

Data are presented as means ± SEM for the number of values indicated in parentheses. A two-factor ANOVA was performed to determine the effects of dose (rhIGF-I and rhIGF-I/IGFBP-3 groups) and treatment (rhIGF-I versus rhIGF-I/IGFBP-3 groups.)

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TABLE 4A  
(page 1 of 2)

Group	Treatment	Day 0			Day 14			Day 28		
		BMD	SEM		BMD	SEM		BMD	SEM	
A	Pretreatment Sham	237	4		ND			ND		
B	Pretreatment OVX	215	4		ND			ND		
1	Sham - Saline	239	5		242	6		243	3	
2	OVX - Saline	216	2		212	3		212	3	
3	0.9 mg/kg IGF-I	211	4		204	5		201	2	
4	2.6 mg/kg IGF-I	214	3		201	4		206	3	
5	0.9 mg/kg IGF-I + IGFBP-3	213	4		210	5		211	4	
6	2.6 mg/kg IGF-I + IGFBP-3	215	5		209	5		204	6	
7	7.5 mg/kg IGF-I + IGFBP-3	219	5		215	4		214	5	

TABLE 4A

		Day 42		Day 56		Change Days 0-56	
		<u>BMD</u>	<u>SEM</u>	<u>BMD</u>	<u>SEM</u>	<u>BMD</u>	<u>SEM</u>
	ND			ND			
	ND			ND			
243	6						
213	3	241	5	3	5		
		208	4	-8	3		
199	4						
202	5	199	5	-12	2		
		201	5	-12	7		
212	4						
206	5	211	4	-2	4		
213	4	210	4	-5	3		
		219	4	0	3		

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b. Bone Measurements

Bone surfaces undergoing active mineralization were labeled in all animals included in this study with the fluorescent bone marker declomycin and calcein by  
5 injecting those markers on days 9 and 2 prior to sacrifice, respectively.

The rats were sacrificed by exsanguination under ketamine/xylazine anesthesia. Numerous soft tissues were collected for histological analyses and some  
10 of these tissues were also weighed to examine organ hypertrophy. The long bones (tibia and femur), the spine and jaws were fixed or frozen for DEXA, mechanical and bone histomorphometric analyses according to the procedures detailed below.

15

1. DEXA Measurements.

Spine DEXA scans were performed on days 0 (described above), 14, 28, 42, and 56 (the day before sacrifice). DEXA whole-body scans (excluding the head  
20 and tail) for measurements of body composition (lean body mass and fat mass) were performed on days 7, 21, 35, and 49.

The DEXA measurements were made with a Hologic QDR-1000/W instrument according to directions provided by  
25 the manufacturer. The rats were anesthetized with a gaseous anesthetic of 2% isoflurane in oxygen during the scans and body weights were also obtained while the rats were anesthetized.

Parameters measured on the tibia and femurs  
30 during the DEXA analyses included length (mm), projected area ("global area") ( $\text{cm}^2$ ), global bone mineral content (BMC) (mg), global bone marrow density (BMD) ( $\text{mg}/\text{cm}^2$ ), global bone mineral apparent density (global BMAD = BMC divided by projected area to the 1.5 power) ( $\text{mg}/\text{cm}^3$ ),  
35 metaphyseal BMD (met BMD) ( $\text{mg}/\text{cm}^2$ ), cortical BMD



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(mg/cm<sup>2</sup>), and epiphyseal BMD (epiph BMD) (mg/cm<sup>2</sup>). The results are shown in Table 5 (tibia) and Table 6 (femur). In these tables, the numerical group designations correspond to those shown in Fig. 1. Groups A and B were  
5 taken for baseline measurements prior to treatment.

DEXA bone measurements were validated by comparing mineral content of the tibia as measured by both global tibia BMC values and ash weight. DEXA body composition measurements were validated by comparing the  
10 changes in body weight measured by both weighing on a balance and DEXA determinations of total body mass.

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Table 5

DEXA Parameters on Excised Tibia																	
Group	Treatment	Length		Global Area		Global BMC		Global BMD		Global BMAD		Met BMD		Cortical BMD		Epiph BMD	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
A	Sham - Pretreatment	39.8	0.4	1.41	0.02	323	7	229	3	193	2	241	3	216	3	287	6
B	OVX - Pretreatment	40.4	0.1	1.39	0.02	286	6	206	2	175	2	187	4	211	3	235	2
1	Sham - Saline	40.3	0.4	1.44	0.04	310	9	221	2	184	2	217	6	209	3	276	5
2	OVX - Saline	40.7	0.6	1.46	0.02	313	7	215	3	178	2	198	3	221	3	231	3
3	0.9 mg/kg IGF-I	40.4	0.4	1.39	0.04	284	12	205	4	174	2	192	5	210	6	217	6
4	2.6 mg/kg IGF-I	41.7	0.4	1.48	0.02	316	7	214	3	176	3	196	5	221	4	226	4
5	0.9 mg/kg IGF-I + IGFBP-3	41.2	0.3	1.46	0.03	306	7	210	2	174	3	197	4	215	3	223	5
6	2.6 mg/kg IGF-I + IGFBP-3	40.8	0.5	1.50	0.04	332	10	223	3	182	3	200	4	227	2	237	4
7	7.5 mg/kg IGF-I + IGFBP-3	42.0	0.2	1.59	0.02	355	4	223	3	177	3	204	3	233	3	238	4

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Table 6

## DEXA Parameters on Excised Femurs

Group	Treatment	Length		Global Area		Global BMC		Global BMD		Global BMAD		Met BMD	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
A	Sham - Pretreatment	36.1	0.3	1.68	0.02	419	10	250	3	193	2	226	4
B	OVX - Pretreatment	35.9	0.3	1.60	0.02	360	7	223	3	175	3	172	5
1	Sham - Saline	36.5	0.3	1.71	0.03	429	15	250	5	191	2	212	5
2	OVX - Saline	36.9	0.3	1.75	0.03	401	10	229	2	173	1	177	3
3	0.9 mg/kg IGF-I	36.2	0.4	1.69	0.04	370	15	218	4	168	2	167	4
4	2.6 mg/kg IGF-I	37.6	0.4	1.78	0.02	411	10	231	3	173	1	173	3
5	0.9 mg/kg IGF-I + IGFBP-3	37.0	0.3	1.76	0.03	394	10	224	4	169	3	169	5
6	2.6 mg/kg IGF-I + IGFBP-3	37.4	0.3	1.80	0.04	424	13	235	4	175	2	174	4
7	7.5 mg/kg IGF-I + IGFBP-3	37.9	0.2	1.86	0.02	451	7	243	2	178	2	174	3

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## 2. Bone Histomorphometry and Structure

Right femurs, tibias and lumbar vertebral bodies (Lvb; L<sub>4</sub>-L<sub>5</sub>) were collected at necropsy, cleaned of soft tissues, fixed in 70% ethanol for 48h, dehydrated through upgraded ethanols, and embedded undecalcified in methyl methacrylate. Longitudinal sections of distal femoral epiphyses, metaphyses, Lvb, and cross-sections of the mid femoral neck and the distal tibial diaphyses proximal to the tibio-fibular junction were cut with a Reichert-Jung supercut microtome (Reichert-Jung, Heidelberg, Germany) to 15  $\mu$ m in thickness. Three consecutive sections were left unstained for dynamic histomorphometric analyses and the other three sections were stained by the von Kossa method for mineral, counterstained with toluidine blue, and analyzed for static histomorphometric parameters. The 100  $\mu$ m thick cross-sections from femoral midshaft and mid-neck region were cut with a precision low-speed bone saw (Isomet, Buehler, Lake Bluff, IL), glued to plastic slides, ground and polished to ~40  $\mu$ m in thickness (Ecomet 3, Buehler, Lake Bluff, IL) and analyzed for bone dynamic parameters. The same set of slides were used for static bone histomorphometry after being stained with the von Kossa method and covered with cover slips. Bone dynamic measurements were performed under UV light and 20-fold magnification by using a semi-automated software program called "Stereology" (KSS Computer Engineers, Magna, UT) described earlier by Miller et al. (1989) Bone 7:283-287. Static bone analyses were performed with an automated television microscope image analysis system and analyzed using "Image Analysis" software (KSS Computer Engineers, Magna, UT).

For the femoral neck only, internal structure of trabecular bone, interconnectivity, and connectivity between endocortical surface and trabecular network, the

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"Nodal" and "Star volume" analyses were performed as described by Miller and Wronski (1993) Anat. Record 236:433-441 and Bagi and Miller (1994) Anat. Record 239:1-12. The marrow star volume is a direct measure of trabecular separation, while the nodal analysis describes the number of nodes and struts, type of struts, and number of inter-trabecular connectivity. Connections between endocortical surface and trabecular network are important determinants of bone strength linking cortical and cancellous bone into one anatomico-functional unit. The total number of endocortico-trabecular connections was counted and the percentages of free ending trabeculae versus those connected with other trabeculae and/or the endocortical surface were calculated. All measurements and derived parameters describing bone dynamic and structure were performed as recommended by ASBMR Histomorphometric Committee (Parfitt et al. (1987) J. Bone Min. Res. 2:595-610.

### 3. Bone Failure Strength and Internal Cortical Bone Structure

Left femurs were cleaned of soft tissue and were used in DEXA measurements of bone mineral density (BMD) and bone mineral content (BMC). To test the biomechanical properties of the cortical bone, the proximal and distal ends of each femur were secured in test fixtures, designed to allow freedom of motion parallel to the long axis of the bone. The fixtures were mounted in an MTS 858 Bionix System (MTS System Corp., Minneapolis, MN) and torque was measured with an externally conditioned miniature torque transducer (range 0-50 in-lbs; Model QWFK-8M/1941, Sensotec, Columbus, OH) as described earlier (van der Meulen, M.C.H., "Bone Strength in Young, Suspended Rats, Stanford University Thesis, 1993). (Mechanical tests were performed at the

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Department of Mechanical Engineering, Stanford University, Stanford, CA; and Rehabilitation Research and Development Center, Palo Alto, CA; and VA Medical Center, Palo Alto, CA). After mechanical testing, the same  
5 specimens were dehydrated in graded ethanols, embedded in methyl methacrylate, and cut with a low speed bone saw (Isomet, Buehler, Lake Bluff, IL) to ~100  $\mu$ m in thickness. The cross-sections (2-3) from the femoral proximal midshafts were glued on plastic slides, ground  
10 and polished to ~40  $\mu$ m thickness and analyzed under polarized light for cortical bone internal structure. The measurements included thickness of the inner and outer lamellar bone layer and thickness of the medially placed woven bone. Following these measurements, samples  
15 were stained by the von Kossa method, counterstained with toluidine blue and cover-slipped. Static bone analyses were performed including the calculation of the second moment of inertia parameter.

#### 20 4. Cellular Bone Analyses

Right tibias were fixed in 10% neutral buffered formalin for 48h, demineralized in Cal-Rite decalcifying solution (Richland-Allan Medical, Richland, MI) for two weeks and processed for paraffin embedding. Three  $\mu$ m  
25 thick longitudinal sections through the proximal tibial metaphysis were stained with hematoxylin and eosin and Gomori's trichrome and were used for cellular bone analyses.

#### 30 5. Statistical Analyses

Differences between plasma glucose values at zero and two hours in Table 3 were analyzed by Student's t-test. A two-factor analysis of variance was employed in Table 3 for comparing differences between treatment  
35 groups. Differences between the groups shown in Tables

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7-16 or Figures 2a-b, 3, 4, 7a-d were tested for significance in a one-way analysis of variance. When the analysis of variance indicated significant differences among means, the differences were evaluated using Dunnett's t-test and Fisher's Protected Least Significant Difference method for multiple comparisons (Netter et al. (1982) "Applied Statistics," Allyn and Bacon, Boston.) Statistical significance was considered at  $P < 0.05$ , and results were expressed as the mean  $\pm$  standard error (SE).

### c. Results

Analysis of data revealed site-specific alterations of cortical and cancellous bone to ovariectomy and treatment with rhIGF-I and rhIGF-I/IGFBP-3 complex. As shown in Table 3, the 2.5 mg/kg dose of rhIGF-I caused acute hypoglycemia which did not occur with any of the doses of rhIGF-I/IGFBP-3. The data presented in Table 4 indicate that no treatment had any influence on glycosylated hemoglobin values and that increasing doses of rhIGF-I and rhIGFBP-3 led to higher serum levels of total IGF-I, rhIGF-I and rhIGFBP-3. Combining rhIGF-I with rhIGFBP-3 resulted in higher serum values of these agents than observed in rats treated with only rhIGF-I.

Spine BMD data throughout the study along with means of the individual changes are presented in Table 4A. As expected, ovariectomy led to a decline in spine BMD which was prevented by the 7.5 mg/kg dose of rhIGF-I/IGFBP-3. Some protection was observed with the two lower doses of rhIGF-I/IGFBP-3 but not with the two doses of rhIGF-I. The DEXA data obtained on excised tibia (Table 5) and femurs (Table 6) show dose-related increases in global BMC and global BMD for both rhIGF-I and rhIGF-I/IGFBP-3.

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### 1. Cortical Bone

Treatment with both formulations tested caused a dose dependent increase in the body weight and in the lean body mass of treated rats (Figs. 2A and 2B).

5 Periosteal bone formation activity was higher in all treated groups and at all sites measured relative to Sham or OVX controls (Tables 7, 8, 9, 11). However, the group of rats treated with 7.5 mg/kg of rhIGF-I/IGFBP-3 complex exhibited the highest increase in the periosteal bone  
10 formation rate parameter (Figs. 4A and 5C, 5CC).

Endocortical bone resorption in treated rats, which is a hallmark of cortical bone changes in estrogen deficient women and rats, were not different from values obtained for the Sham-op controls (Fig. 4B), showing  
15 significantly lower values compared to OVX rats at the femoral neck location (Table 11). Also, bone formation at the endocortical envelope was increased at both bone sites measured (femoral diaphyses, Table 8).

Internal structural analyses showed normal  
20 "lamellar" configuration of the bone formed on both cortical envelopes after rhIGF-I/IGFBP-3 treatment (Table 9; Fig. 6). Increased modeling-dependent bone formation on the periosteal and endocortical bone envelopes and moderate or decreased bone resorption on the endocortical  
25 envelope resulted in thicker cortical bone in the treated rats (Table 7, 9, 11; Figs. 5 and 6). Increased cortical thickness and newly formed lamellar bone which is mechanically superior to woven bone resulted in the higher failure torque and polar moment of inertia  
30 parameters in rats treated with 7.5 mg/kg of rhIGF-I/IGFBP-3 complex (Table 10).

All treated animals exhibited similar increases in longitudinal bone growth when compared to OVX of Sham rats (Fig. 3). In general, treatment with rhIGF-I/  
35 IGFBP-3 caused cortical bone thickening by adding



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"lamellar" bone on both envelopes. The increase in lean body component might potentiate periosteal bone formation by increasing the muscle pull on the periosteum. Both "lamellar" structure and increased cortical thickness improved cortical bone strength after rhIGF-I/IGFBP-3 treatment.

## 2. Cancellous Bone

Cancellous bone dynamics and structure were examined at four different locations in the rat skeleton. Differences in different locations in the bone turnover rates and functional anatomy between the designated sites (distal femoral epiphysis, distal femoral metaphysis, femoral mid-neck, and lumbar vertebral bodies) were measured. Treatment with rhIGF-I/IGFBP-3 increased bone formation parameters at all four sites (Tables 12, 13, 14, 15; Figs. 7 and 8). The increase in bone formation rates (volume referents; Figs. 7A-7D) was the highest in the group treated with 7.5 mg/kg of rhIGF-I/IGFBP-3 complex. Bone resorption had similar or lower values compared to OVX rats (Tables 12-15). Such turnover activity resulted in increased trabecular thickness and preservation of the trabecular number in treated rats at all sites measured, except in the femoral metaphysis.

Primary and secondary spongiosa at the femoral metaphyseal site was highly affected by the endochondral bone elongation, particularly in the OVX rats and after treatment with rhIBF or rhIBF-I/IBFBP-3 (Figs. 9A-9C). This bone site has the highest bone turnover rate. OvX caused disappearance of "metabolic" trabeculae in the central metaphyseal region with no mechanical value (Fig. 9B) while treatment with rhIGF-I/IGFBP-3 complex helped the restoration (Fig. 9C).

Structural trabecular bone analyses were performed at the femoral neck location, which is the most

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relevant bone site for fractures in humans (Table 16;  
Figs. 10A-10C). Besides having a very unique anatomy,  
this bone site combines cortical and cancellous bone  
interconnected at the endocortical surface. Cortical  
5 bone thickness, cancellous bone mass, structure, and  
connectivity between endocortical surface and trabecular  
network are all important determinants of the bone  
strength in the femoral neck region. The treatment with  
the rhIGF-I/IGFBP-3 improved trabecular bone structure  
10 and connectivity between the trabecular network and  
endocortical surface. In general, data presented here  
revealed benefits of systemically administered rhIBF-I/  
IBFBP-3 on the entire musculo-skeletal system in  
previously ovariectomized rats.

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**Table 7:** Cortical bone morphometry measured at the tibio-fibular junction in rats treated with rhIGF-I alone, or with equimolar doses of rhIGF-I/IGFBP-3 complex for eight weeks.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)				rhIGF-I (mg/kg)+rhIGFBP-3			
			0.9	2.6	0.9	2.6	0.9	2.6	0.9	2.6
Periosteal perimeter (mm)	8.64±0.12	9.24±0.15†	9.19±0.18†	9.29±0.27†	9.21±0.21†	9.43±0.17†	9.21±0.21†	9.43±0.17†	9.21±0.21†	9.43±0.17†
Endocortical perimeter (mm)	3.56±0.06	4.03±0.18†	3.8±0.15	4.12±0.2†	3.73±0.14	3.69±0.09b	3.73±0.14	3.69±0.09b	3.73±0.14	3.69±0.09b
Organ area (mm <sup>2</sup> )	5.52±0.09	5.69±0.18†	5.59±0.27	5.83±0.24†	5.63±0.18	5.78±0.14*	5.63±0.18	5.78±0.14*	5.63±0.18	5.78±0.14*
Marrow area (mm <sup>2</sup> )	0.95±0.02	1.08±0.05†	0.92±0.09	1.03±0.06†	0.67±0.05†	0.81±0.05*	0.67±0.05†	0.81±0.05*	0.67±0.05†	0.81±0.05*
Cortical area (mm <sup>2</sup> )	4.57±0.07	4.62±0.13†	4.68±0.29*	4.8±0.17†	4.95±0.14†	4.97±0.13†	4.95±0.14†	4.97±0.13†	4.95±0.14†	4.97±0.13†
Percent periosteal double labeled surface (%)	11.76±4.6	10.33±2.9†	43.09±8.4†	67.27±9.6†	44.12±3.8†	73.09±4.5†	44.12±3.8†	73.09±4.5†	44.12±3.8†	73.09±4.5†
Periosteal new bone area (μm <sup>2</sup> )	0.004±0.002	0.002±0.001†	0.02±0.01†	0.03±0.01†	0.02±0.004†	0.07±0.01†,b	0.02±0.004†	0.07±0.01†,b	0.02±0.004†	0.07±0.01†,b
Periosteal mineralized surface (%)	12.16±4.7	8.33±3.2†	43.52±8.8†	52.29±9.0†	44.68±4.1†	63.65±5.5†	44.68±4.1†	63.65±5.5†	44.68±4.1†	63.65±5.5†
Periosteal mineral appositional rate (μm/d)	0.33±0.17	0.17±0.12†	0.71±0.05†	1.03±0.09†	0.62±0.04†	1.42±0.1†,b	0.62±0.04†	1.42±0.1†,b	0.62±0.04†	1.42±0.1†,b
Periosteal bone formation rate (μm <sup>2</sup> /μm/d)	0.06±0.03	0.02±0.02	0.27±0.08†	0.4±0.11†	0.21±0.04†	0.77±0.11†,b	0.21±0.04†	0.77±0.11†,b	0.21±0.04†	0.77±0.11†,b
Periosteal bone formation rate (%/y)	5.22±2.18	1.63±3.65	18.39±14.2†	28.8±8.78†	13.67±6.8†	53.7±13.8†,b	13.67±6.8†	53.7±13.8†,b	13.67±6.8†	53.7±13.8†,b

Data are expressed as the mean ± SE; † Significantly different from Sham group, P<0.05 by Dunnett's test; \* Significantly different from OvX group, P<0.05 by Dunnett's test; b Significantly different from rhIGF-I (2.6 mg/kg) group, P<0.05 by Fisher's PLSD test.

**Table 8:** Dynamic cortical bone parameters measured at the femoral diaphyses in control rats and rats treated with 7.5mg/kg of rhIGF-I/IGFBP-3 complex for eight weeks.

<u>Parameter</u>	<u>Basal Sham</u>	<u>Basal Ovx</u>	<u>Sham</u>	<u>Ovx</u>	<u>IGF-I/IGFBP-3</u>
Periosteal mineralizing surface (%)	50.18±5.26	58.34±3.15	41.38±3.66 <sup>b</sup>	32.87±4.05 <sup>a,b</sup>	86.45±4.72 <sup>a,b,c,d</sup>
Periosteal mineral appositional rate (µm/d)	0.61±0.02	1.03±0.04 <sup>a</sup>	0.76±0.05 <sup>a,b</sup>	0.83±0.04 <sup>a,b</sup>	1.29±0.07 <sup>a,b,c,d</sup>
Periosteal bone formation rate (µm <sup>2</sup> /µm/d)	0.23±0.03	0.49±0.02 <sup>a</sup>	0.26±0.03 <sup>b</sup>	0.2±0.04 <sup>b</sup>	1.07±0.13 <sup>a,b,c,d</sup>
Periosteal bone formation rate (%/y)	21.27±2.5	45.85±0.9 <sup>a</sup>	24.89±1.48 <sup>b</sup>	21.4±3.02 <sup>b</sup>	88.81±10.3 <sup>a,b,c,d</sup>
Endocortical mineralizing surface (%)	1.02±0.43	4.03±0.69	1.69±0.27	2.14±0.4 <sup>c</sup>	17.79±4.22 <sup>a,b,c,d</sup>
Endocortical mineral appositional rate (µm/d)	0.009±0.01	0.425±0.19 <sup>a</sup>	0.068±0.06 <sup>b</sup>	0.052±0.05 <sup>b</sup>	0.807±0.13 <sup>a,b,c,d</sup>
Endocortical bone formation rate (µm <sup>2</sup> /µm/d)	0.008±0.05	0.023±0.01	0.002±0.001	0.001±0.001	0.173±0.046 <sup>a,b,c,d</sup>
Endocortical bone formation rate (%/y)	0.04±0.02	2.16±0.86	0.42±0.42	0.32±0.32	7.79±2.35 <sup>a,b,c,d</sup>
Endocortical resorption surface (%)	1.01±0.25	4.14±0.8 <sup>a</sup>	1.79±0.23 <sup>b</sup>	6.33±0.79 <sup>a,b,c</sup>	2.71±0.33 <sup>a,d</sup>

Data are expressed as the mean ± SE; Significant difference P<0.05 by Fisher's PLSD test.

<sup>a</sup> Significantly different from Basal Sham group; <sup>b</sup> Significantly different from Basal Ovx group;

<sup>c</sup> Significantly different from Sham group; <sup>d</sup> Significantly different from Ovx group.

**Table 9:** Static cortical parameters measured at the femoral midshafts, and structural parameters measured at the postero-lateral 1/4 of the cross-sectional areas in control rats and rats treated with 7.5mg/kg of rhIGF-1/IGFBP-3 complex for eight weeks.

<u>Parameter</u>	<u>Basal Sham</u>	<u>Basal Ovx</u>	<u>Sham</u>	<u>Ovx</u>	<u>IGF-1/IGFBP-3</u>
Periosteal					
perimeter (mm)	9.54±0.09	9.65±0.23	9.76±0.11	10.51±0.19a,b,c	11.61±0.41a,b,c,d
Endocortical					
perimeter (mm)	5.4±0.13	5.79±0.18	5.38±0.12	5.98±0.18a,c	5.69±0.15
Organ					
area (mm <sup>2</sup> )	6.02±0.13	5.89±0.22	6.05±0.16	6.65±0.19a,b,c	7.03±0.15a,b,c
Marrow					
area (mm <sup>2</sup> )	1.76±0.05	2.0±0.13	1.82±0.09	2.18±0.13a,c	1.86±0.09d
Cortical					
area (mm <sup>2</sup> )	4.26±0.09	3.89±0.1	4.23±0.12	4.46±0.09b	5.17±0.09a,b,c,d
Average cortical					
width (μm)	601.4±7.9	568.6±1.5a	597.5±6.1b	574.8±3.8a,c	614.8±5.6b,c,d
Percent cortical					
area (%)	70.7±0.5	69.16±0.77	69.9±1.1	67.3±1.2a	73.6±0.9a,b,c,d
<u>Structural parameters:</u>					
Outer lamellar					
layer (μm)	277.1±8.9	273.5±8.4	274.5±11.3	316.8±14.6a,b,c	280.3±10.1d
Central woven					
layer (μm)	119.8±5.1	114.6±6.0	120.5±6.4	103.7±14.5	114.2±6.2
Inner lamellar					
layer (μm)	213.3±8.1	145.4±11.2a	202.6±9.2b	70.4±8.8a,b,c	242.6±13.7a,b,c,d

Data are expressed as the mean ± SE; Significant difference  $P < 0.05$  by Fisher's PLSD test.

a Significantly different from Basal Sham group; b Significantly different from Basal Ovx group; c Significantly different from Sham group; d Significantly different from Ovx group.

**Table 10:** Femoral failure strength and general parameters in control rats and rats treated with 7.5mg/kg of rhIGF-I/IGFBP-3 complex for eight weeks.

<u>Parameter</u>	<u>Basal Sham</u>	<u>Basal Ovx</u>	<u>Sham</u>	<u>Ovx</u>	<u>IGF-I/IGFBP-3</u>
Failure torque (N/mm)	461.9±526.8	387.0±41.1 <sup>a</sup>	390.6±31.2	443.6±21.2 <sup>b,c</sup>	471.5±15.3 <sup>b,c,d</sup>
Torsional rigidity (Ncm <sup>2</sup> /rad)	454±58	439±66	468±98	479±93	468±39
Linear stiffness (N-m/degrees)	43.4±2.6	40.7±2.5	42.6±2.5	43.1±3.9	44.9±2.8
Polar moment inertia (mm <sup>4</sup> )	6.6±0.7	6.3±0.9	6.5±0.6	6.8±1.3 <sup>b</sup>	9.3±1.8 <sup>a,b,d</sup>
Femoral length (mm)	36.0±0.3	35.8±0.3	36.6±0.4	36.8±0.4 <sup>b</sup>	37.9±0.2 <sup>a,b,c,d</sup>
Femoral area (cm <sup>2</sup> )	1.67±0.03	1.66±0.04	1.73±0.03	1.74±0.04	1.86±0.02 <sup>a,b,c,d</sup>
Femoral BMC (g)	0.41±0.01	0.37±0.01 <sup>a</sup>	0.44±0.02 <sup>b</sup>	0.34±0.01 <sup>c</sup>	0.45±0.01 <sup>a,b,d</sup>
Femoral BMD (g/cm <sup>2</sup> )	0.25±0.003	0.23±0.004 <sup>a</sup>	0.25±0.01 <sup>b</sup>	0.23±0.003 <sup>a,c</sup>	0.24±0.002 <sup>b,d</sup>

Data are expressed as the mean ± SE; Significant difference  $P < 0.05$  by Fisher's PLSD test.

<sup>a</sup> Significantly different from Basal Sham group; <sup>b</sup> Significantly different from Basal Ovx group;

<sup>c</sup> Significantly different from Sham group; <sup>d</sup> Significantly different from Ovx group.

**Table 11:** Cortical bone morphometry measured at femoral neck cross-sectional areas after eight weeks of treatment with rhIGF-I alone or rhIGF-I/IGFBP-3 complex.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)		rhIGF-I (mg/kg)+equimolar rhIGFBP-3	
			0.9	2.6	0.9	2.6
<u>Entire organ</u>						7.5
Cortical bone						
area (%)	83.3±1.4	78.9±1.7*	84.3±1.0 <sup>ⓐ</sup>	84.9±1.3 <sup>ⓐ</sup>	84.1±1.5 <sup>ⓐ</sup>	87.3±1.4 <sup>ⓐ</sup>
Bone marrow						
area (%)	13.9±0.1	13.1±0.1*	8.4±0.1** <sup>ⓐ</sup>	6.4±0.1** <sup>ⓐ</sup>	7.8±0.1** <sup>ⓐ</sup>	7.9±0.1** <sup>ⓐ</sup>
Average cortical						
width (μm)	472±9	447±8	465±11	471±12	453±12	466±14
<u>Periosteal envelope</u>						
Mineralized						
surface (%)	4.2±0.4	7.1±1.3*	7.9±1.4*	11.8±1.0** <sup>ⓐ</sup>	8.0±0.9*	9.7±1.7*
Mineral appositional						
rate (μm/d)	0.24±0.01	0.24±0.01	0.38±0.03** <sup>ⓐ</sup>	0.48±0.02** <sup>ⓐ</sup>	0.36±0.01** <sup>ⓐ</sup>	0.35±0.01** <sup>ⓐ</sup>
Bone formation						
rate (μm <sup>2</sup> /μm/d)	0.01±0.001	0.01±0.003	0.02±0.01*	0.04±0.01** <sup>ⓐ</sup>	0.02±0.003*	0.02±0.01** <sup>ⓐ</sup>
Bone formation						
rate (%/y)	7.3±0.8	9.6±1.3	34.7±5.2** <sup>ⓐ</sup>	55.4±4.5** <sup>ⓐ</sup>	37.47±6.1** <sup>ⓐ</sup>	37.0±5.8** <sup>ⓐ</sup>
						59.0±6.2† <sup>ⓐ,c</sup>

Table 11 (cont'd): Cortical bone morphometry measured at femoral neck cross-sectional areas after eight weeks of treatment with rhIGF-I alone or rhIGF-I/IGFBP-3 complex.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)	rhIGF-I (mg/kg)+equimolar rhIGFBP-3
	0.9		2.6	0.9 2.6 7.5
<i>Endocortical envelope</i>				
Mineralized surface (%)	6.5±0.6	8.8±1.3	12.2±1.5 <sup>a</sup>	11.7±1.6 <sup>a</sup> 19.7±0.9 <sup>a,ⓐ</sup> 17.9±2.3 <sup>a,ⓑ</sup> 27.6±2.4 <sup>a,ⓐ,c</sup>
Mineral appositional rate (μm/d)	0.23±0.01	0.24±0.01	0.49±0.02 <sup>a</sup>	0.49±0.03 <sup>a</sup> 0.6±0.01 <sup>a,ⓐ</sup> 0.53±0.03 <sup>a</sup> 0.57±0.02 <sup>a,ⓐ,c</sup>
Bone formation rate (μm <sup>2</sup> /μm/d)	0.01±0.001	0.01±0.002	0.05±0.02 <sup>a</sup>	0.04±0.01 <sup>a</sup> 0.07±0.01 <sup>a,ⓐ</sup> 0.08±0.01 <sup>a,ⓑ</sup> 0.1±0.01 <sup>a,ⓐ,c</sup>
Bone formation rate (%/y)	3.2±0.3	5.8±1.1	27.8±4.7 <sup>a</sup>	27.4±5.4 <sup>a</sup> 46.7±6.9 <sup>a,ⓐ</sup> 60.6±11.1 <sup>a,ⓑ</sup> 79.9±8.5 <sup>a,ⓐ,c</sup>
Resorption perimeter (%)	4.8±0.2	7.5±0.6 <sup>a</sup>	5.2±0.6 <sup>ⓐ</sup>	3.9±0.4 <sup>a</sup> 4.8±0.4 <sup>a</sup> 3.9±0.5 <sup>ⓐ,ⓑ</sup> 3.2±0.3 <sup>a,ⓐ,c</sup>

Data are expressed as the mean ± SE; <sup>a</sup>P<0.05 Significantly different from Sham group; <sup>ⓐ</sup>P<0.05 Significantly different from Ovx group; <sup>ⓑ</sup>P<0.05 Significantly different from rhIGF-I (0.9 mg/kg) group; <sup>c</sup>P<0.05 Significantly different from rhIGF-I (2.6 mg/kg) group; <sup>ⓐ,c</sup>P<0.05 Significantly different from rhIGF-I/IGFBP-3 (0.9 mg/kg and/or 2.6 mg/kg) group.



Table 12: Cancellous bone morphometry measured at distal femoral metaphysis after eight weeks of treatment with rhIGF-I alone or rhIGF-I/IGFBP-3 complex.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)				rhIGF-I (mg/kg)+equimolar rhIGFBP-3			
			0.9	2.6	0.9	2.6	0.9	2.6	0.9	2.6
Percent double labeled perimeter (%)	6.05±0.64	17.41±1.56†	14.21±0.77†	17.32±1.33†	18.02±0.84†	16.23±1.11†	16.23±1.11†	26.61±2.26† <sup>a</sup>		7.5
Percent resorption perimeter (%)	5.89±0.3	11.01±1.46†	9.61±0.67†	14.01±2.07†	9.08±1.01†	10.16±0.56† <sup>b</sup>	10.16±0.56† <sup>b</sup>	14.45±0.97† <sup>a</sup>		
Corrected mineral appositional rate (µm/d)	0.17±0.03	0.47±0.06†	0.38±0.03†	0.41±0.03†	0.44±0.02†	0.44±0.03†	0.44±0.03†	0.48±0.04†		
Mineralized surface (%)	2.52±0.36	8.54±1.5†	5.35±1.79† <sup>a</sup>	6.35±2.44†	10.48±3.24† <sup>a</sup>	13.56±3.53†	13.56±3.53†	19.89±4.09† <sup>a</sup>		
Bone formation rate (µm <sup>2</sup> /µm/d)	0.001±0.001	0.021±0.006†	0.014±0.001†	0.012±0.002†	0.015±0.002†	0.014±0.002†	0.014±0.002†	0.036±0.002† <sup>a</sup>		
Endochondral growth (µm/d)	3.77±0.21	3.81±0.29	5.57±0.33† <sup>a</sup>	6.44±0.4† <sup>a</sup>	5.89±1.33† <sup>a</sup>	5.89±0.26† <sup>a</sup>	5.89±0.26† <sup>a</sup>	6.15±0.28† <sup>a</sup>		
Percent trabecular area (%)	25.02±1.58	14.95±0.92†	19.48±0.78† <sup>a</sup>	14.73±1.33†	16.15±0.99† <sup>a</sup>	17.86±1.41†	17.86±1.41†	11.64±0.9† <sup>a</sup>		
Trabecular thickness (µm)	52.35±2.44	58.8±1.29†	66.24±0.58†	56.37±1.88	55.11±1.59	68.2±1.2† <sup>a,b</sup>	68.2±1.2† <sup>a,b</sup>	50.0±2.12 <sup>a</sup>		
Trabecular number (No/mm)	4.78±0.21	2.53±0.12†	2.97±0.1† <sup>a</sup>	2.54±0.17†	2.85±0.13†	2.74±0.13†	2.74±0.13†	2.3±0.15†		
Separation (µm)	229.8±12.7	492.69±26.2†	393.9±16.9† <sup>a</sup>	501.1±39.2†	429.8±22.4†	447.3±37.1†	447.3±37.1†	571.7±43.7†		
Perimeter/Area ratio (mm/mm <sup>2</sup> )	32.43±1.54	28.48±0.64†	25.38±0.37† <sup>a</sup>	29.21±1.0	29.7±0.89 <sup>a</sup>	25.9±0.8† <sup>a,b</sup>	25.9±0.8† <sup>a,b</sup>	33.23±0.83		

Data are expressed as the mean ± SE; † Significantly different from Sham group, P<0.05 by analysis of variance followed by Dunnett's test; <sup>a</sup> Significantly different from Ovx group, P<0.05 by analysis of variance followed by Dunnett's test;

<sup>b</sup> Significantly different from rhIGF-I (0.9 mg/kg) group, P<0.05 by analysis of variance followed by Fisher's PLSD method;

<sup>c</sup> Significantly different from rhIGF-I (2.6 mg/kg) group, P<0.05 by analysis of variance followed by Fisher's PLSD method.

**Table 13:** Cancellous bone morphometry measured at distal femoral epiphysis after eight weeks of treatment with rhIGF-I alone or rhIGF-I/IGFBP-3 complex.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)		rhIGF-I (mg/kg)+equimolar rhIGFBP-3		
			0.9	2.6	0.9	2.6	7.5
Percent double							
labeled perimeter (%)	6.07±0.49	18.72±2.02†	9.35±0.62†*	13.43±1.45†*	22.89±1.32†.a	24.12±2.06†.b	28.24±1.36†*
Percent resorption							
perimeter (%)	5.93±0.48	7.18±0.57	5.11±0.39*	5.26±0.54*	4.73±0.49*	5.19±0.33*	7.13±0.52
Corrected mineral							
appositional rate (µm/d)	0.17±0.05	0.51±0.04†	0.57±0.01†	0.39±0.04†*	0.55±0.02†	0.58±0.07†.b	0.7±0.02†*
Mineralized							
surface (%)	1.56±0.09	9.65±2.58†	6.01±2.12†*	9.28±3.47†	12.31±3.2†*.a	17.71±4.05†.b	21.39±3.87†*
Bone formation							
rate (µm <sup>2</sup> /µm/d)	0.001±0.003	0.016±0.004†	0.008±0.001†	0.008±0.002†	0.016±0.003†	0.023±0.005†.b	0.035±0.003†*
Percent trabecular							
area (%)	37.19±1.44	31.01±0.74†	41.28±2.06*	37.8±1.67*	36.3±1.54*.a	34.11±0.69	40.11±1.73*
Trabecular							
thickness (µm)	83.9±2.6	82.68±3.0	92.43±4.13	96.5±2.87†*	92.04±2.33†*	92.01±0.98†*	98.79±4.13†*
Trabecular							
number (No/mm)	4.33±0.16	3.79±0.16†	4.45±0.14*	3.71±0.13†	4.42±0.2*	3.89±0.1	4.07±0.17
Trabecular							
separation (µm)	206.4±11.8	265.5±11.6†	191.9±11.9*	241.1±10.9†	207.1±11.9*	240.0±5.7†*	210.9±10.9*
Perimeter/Area							
ratio (mm/mm <sup>2</sup> )	20.03±0.61	20.43±0.89	18.2±0.76	16.52±0.97†*	19.17±1.15	18.45±0.41	17.06±0.84†*

Data are expressed as the mean ± SE; † Significantly different from Sham group, P<0.05 by analysis of variance followed by Dunnett's test;

\* Significantly different from Ovx group, P<0.05 by analysis of variance followed by Dunnett's test;

a Significantly different from rhIGF-I (0.9 mg/kg) group, P<0.05 by analysis of variance followed by Fisher's PLSD method;

b Significantly different from rhIGF-I (2.6 mg/kg) group, P<0.05 by analysis of variance followed by Fisher's PLSD method.

Table 14: Cancellous bone morphometry measured at lumbar vertebral bodies (L4-L5) after eight weeks of treatment with rhIGF-I alone or rhIGF-I/IGFBP-3 complex.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)		rhIGF-I (mg/kg)+equimolar rhIGFBP-3		
			0.9	2.6	0.9	2.6	7.5
Percent double labeled perimeter (%)	8.17±0.99	14.2±1.16†	11.23±0.68	14.31±1.47†	12.0±0.79†	14.12±1.3†	16.06±1.21†
Percent resorption perimeter (%)	6.19±0.48	8.88±0.97†	5.37±0.59*	10.1±1.26†	6.46±0.57	11.34±0.99†	8.57±0.71†
Corrected mineral appositional rate (µm/d)	0.13±0.04	0.33±0.04†	0.4±0.01†	0.32±0.03†	0.29±0.03†,a	0.35±0.02†	0.35±0.04†
Mineralized surface (%)	2.17±0.84	7.38±2.46†	6.28±1.25†	7.86±1.68†	7.21±2.15†	8.56±2.03†	11.97±4.37†
Bone formation rate (µm <sup>2</sup> /µm/d)	0.001±0.001	0.008±0.002†	0.009±0.001†	0.007±0.002†	0.011±0.001†	0.007±0.002†	0.013±0.003†
Percent trabecular area (%)	36.53±1.89	28.46±1.32†	30.06±1.32†	35.04±0.97*	32.95±1.46*	34.11±2.99*	37.65±1.57*
Trabecular thickness (µm)	57.73±2.56	57.26±1.58	58.62±0.99	65.87±3.01†,*	63.66±3.34†,*	64.44±3.5†,*	66.42±2.51†,*
Trabecular number (No/mm)	6.32±0.09	4.96±0.17†	5.12±0.18†	5.37±0.13*	5.2±0.15	5.25±0.24	5.67±0.09*
Trabecular separation (µm)	145.1±6.05	210.2±11.1†	198.8±10.4†,*	174.8±3.15*	186.2±6.7*	185.0±15.0*	158.7±16.0*
Perimeter/Area ratio (mm/mm <sup>2</sup> )	29.51±0.36	29.32±0.84	28.51±0.48	25.69±1.16†,*	26.74±1.46	26.46±1.52	25.39±1.05†,*

Data are expressed as the mean ± SE; † Significantly different from Sham group, P<0.05 by analysis of variance followed by Dunnett's test.

\* Significantly different from Ovx group, P<0.05 by analysis of variance followed by Dunnett's test.

<sup>a</sup> Significantly different from rhIGF-I (0.9 mg/kg) group, P<0.05 by analysis of variance followed by Fisher's PLSD method;

<sup>b</sup> Significantly different from rhIGF-I (2.6 mg/kg) group, P<0.05 by analysis of variance followed by Fisher's PLSD method.

Table 15: Cancellous bone morphometry measured at femoral neck cross-sectional areas after eight weeks of treatment with rhIGF-I alone or rhIGF-I/IGFBP-3 complex.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)		rhIGF-I (mg/kg)+equimolar rhIGFBP-3	
			0.9	2.6	0.9	2.6
Percent resorption						
perimeter (%)	4.5±0.2	10.1±0.8*	6.1±0.5 <sup>Ⓢ</sup>	5.2±0.4 <sup>Ⓢ</sup>	6.1±0.6 <sup>Ⓢ</sup>	6.9±0.7* <sup>Ⓢ</sup>
Corrected mineral appositional rate (μm/d)	0.23±0.01	0.32±0.01*	0.52±0.04* <sup>Ⓢ</sup>	0.54±0.01* <sup>Ⓢ</sup>	0.5±0.04* <sup>Ⓢ,a</sup>	0.52±0.02* <sup>Ⓢ,b</sup>
Mineralized surface (%)	5.8±0.7	11.9±1.3*	12.3±0.9*	13.8±0.9*	14.9±1.4*	15.9±2.6*
Bone formation rate (μm <sup>2</sup> /μm/d)	0.001±0.001	0.006±0.002	0.012±0.002*	0.014±0.003*	0.014±0.003*	0.015±0.003*
Percent trabecular area (%)	12.8±1.9	8.1±0.6*	7.4±1.1*	8.6±1.3*	8.1±1.3*	7.4±0.7*
Trabecular thickness (μm)	83.5±6.2	69.5±5.4	79.9±2.6	75.1±3.8	69.3±8.6	78.3±2.2
Trabecular number (No/mm)	10.8±1.4	5.6±0.5*	6.9±0.9*	8.1±1.4	7.8±0.6*	5.3±0.6*
Trabecular separation (μm)	119.4±12.6	263.3±17.1*	206.8±15.2* <sup>Ⓢ</sup>	188.3±24.6* <sup>Ⓢ</sup>	183.1±20.3* <sup>Ⓢ</sup>	186.1±10.3* <sup>Ⓢ</sup>
Perimeter/Area ratio (mm/mm <sup>2</sup> )	15.7±1.6	23.9±2.5*	19.6±0.8	20.7±1.0	20.0±2.2	18.3±1.3
						21.9±2.6

Data are expressed as the mean ± SE; \*P<0.05 Significantly different from Sham group; <sup>Ⓢ</sup>P<0.05 Significantly different from Ovx group; aP<0.05 Significantly different from rhIGF-I (0.9 mg/kg) group; bP<0.05 Significantly different from rhIGF-I (2.6 mg/kg) group; cP<0.05 Significantly different from rhIGF-I/IGFBP-3 (0.9 mg/kg and/or 2.6 mg/kg) group.

**Table 16:** Structural analyses of cancellous bone performed on von Kossa stained cross-sections of the femoral neck from rats treated for 8 weeks with rhIGF-I alone or the rhIGF-I/IGFBP-3 complex. A nodal analysis was employed for trabecular and endocortico-trabecular connectivities and star volume analysis for trabecular separation.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)		rhIGF-I (mg/kg)+equimolar rhIGFBP-3	
			0.9	2.6	0.9	2.6
<u>Nodal analysis</u>						
Number of nodes	12.9±1.7	6.1±0.5*	5.5±0.3*	5.9±0.3*	7.0±0.7*	6.5±0.3*
Number of struts	16.0±1.3	9.9±0.4*	7.3±0.4*,a	8.5±0.6*	8.3±0.7*	9.3±0.6*
<u>Type of strut (% of total)</u>						
Free-free	5.8±1.2	20.6±1.8*	5.3±2.6a	8.7±2.9a	6.8±1.9a	7.1±2.8a
Node-free	18.9±1.7	39.6±2.2*	29.8±3.6*	31.3±2.5*	21.2±5.4a,b	24.3±1.2a
Node-node	75.4±2.0	39.7±3.1*	64.9±4.8*,a	61.7±3.1*,a	68.6±6.2a	66.4±2.4a
<u>Endocortico-trabecular connectivity</u>						
Total number of endocortico-trabecular connections	11.6±0.9	6.8±0.5*	6.0±0.5*	6.4±0.5*	7.8±0.7*	6.9±0.6*
Endocortico-endocortical (%)	95.1±1.2	42.3±1.4	50.2±2.4*	53.2±2.3*,a	54.3±1.7*,a	55.1±2.3*,a
Endocortico-free ending (%)	4.9±0.3	57.7±1.4	49.8±2.1*	46.8±2.0*,a	45.7±1.3*,a	44.9±2.1*,a
<u>Star volume analysis</u>						
Marrow volume (mm <sup>3</sup> )	0.01±0.001	0.03±0.004*	0.01±0.002a	0.013±0.005a	0.01±0.002a	0.006±0.001a

Data are expressed as the mean ± SE; \*P<0.05 Significantly different from Sham group; <sup>a</sup>P<0.05 Significantly different from rhIGF-I (0.9 mg/kg) group; <sup>b</sup>P<0.05 Significantly different from rhIGF-I (2.6 mg/kg) group; <sup>c</sup>P<0.05 Significantly different from rhIGF-I/IGFBP-3 (0.9 mg/kg and/or 2.6 mg/kg) group.

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Example 3Recombinant synthesis of IGF-I

Recombinant rhIGF-I was produced using a gene insert coding for the sequence shown in Figure 11 (SEQ ID NO:1), as detailed below.

Materials

The bacterial strain used is a derivative of Escherichia coli K-12 strain W3110 which has been lysogenized with DE3. (Studier, F. and Moffat, B. [1986] J. Mol. Biol. 189: 113-130). This lysogen carries the gene for T7 RNA polymerase under the control of the lacUV5 promoter.

This host strain was transformed with plasmids pER10088 by selection for tetracycline-resistance.

Description of Plasmids

The three expression vectors used in this work are similar to pJU1002 and pJU1003 (Squires, C.H., et al. [1988] J. Biol. Chem. 263: 16297-16302) except that the genes inserted downstream of the translational coupler are ubiquitin-IGF (pER10088). In addition, pER 10088 differs from pJU1003 in that it does not contain the synthetic 16 bp adaptor sequence at the 5' end of the tet gene in that plasmid; however, it does contain DNA insertions at the unique PvuII site in the pBR322-derived backbone: pER10088 contains a linker 5'...CCTCGAGG...3' at that location.

Description of Gene Inserts

As produced for studies carried out in support of the present invention, the vector pER10088 contained an open reading frame (ORF) comprising (in order 5' to 3') an ATG triplet (initiation), the 76 codons of yeast ubiquitin, 70 synthetic codons of mature human insulin

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growth factor I (Figure 11), and a termination codon. In this case, the ORF is positioned relative to the translational coupler exactly as described by Squires et al. (above) for fibroblast growth factor.

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#### IGF purification

E. coli cells producing IGF-I were broken open using a Gaulin mill or Microfluidizer in 5 volumes/wt (e.g., 1 l/200 g cell paste) of 50 mM sodium acetate and 1 mM EDTA, pH 5.5 while its temperature was maintained at not higher than 10°C. The lysate was checked for the presence of whole E. coli cells using a light microscope. To the lysate was added one-hundredth volume of a 10% (w/v) solution of polyethyleneimine and then centrifuged at 10,000 for 20 minutes to separate supernatant from pellet. The pellet was re-extracted twice more in 2.5 volumes of 20 mM potassium phosphate, 20 mM DTT and 2 M urea pH 5.8 and centrifuged as above. The pellet was used for further purification steps.

IGF was extracted from the pellet with 5 volumes/wt of 20 mM Tris pH 8.0 containing 6 M urea, 40 mM DTT and 1 mM EDTA and filtered through a Sartorius 0.8  $\mu$  filter. The filtrate was diluted with the same buffer to a final protein concentration of 3 mg/ml, then diluted with 2 volumes of 20 mM Tris and 1 mM EDTA pH 8.0. This mixture was subjected to a DNA removal step using protamine sulfate and Q-Sepharose, according to standard methods. Ubiquitin protein peptidase was added to the mixture to release IGF-I from the ubiquitin IGF-I fusion protein, and IGF-I was refolded overnight at ambient temperature. A typical refolding reaction was carried out at about 1 mg/ml protein concentration, 1.5 to 2 M urea and pH range 8-9 in presence of Tris buffer at 20 to 50 mM and a ratio of DTT/Cystamine of 1. Refolding performed as above yields about 40% of the

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correctly refolded IGF-I compared to the initial amount of IGF-I.

The refolded IGF-I solution was clarified, buffer was exchanged using ultrafiltration system and loaded onto a cation exchange column equilibrated in 50 mM sodium acetate buffer, pH 5.5. Following the loading of the IGF-I solution onto the column, the column was washed with the equilibration buffer and purified IGF-I was then eluted from the column by application of 20 column volumes of gradient starting with equilibration buffer and finishing with either 0.25 or 0.5 M sodium chloride. The appropriate fractions of pure IGF-I were pooled.

Pooled fractions from the cation exchange column were acidified to pH 2.5 with 10% trifluoroacetic acid (TFA). The solution was filtered through a 0.2  $\mu$ m filter and loaded onto a Vydac C-4 column equilibrated in 0.1% TFA in water. The column was developed with a linear gradient of 0 to 40% of acetonitrile in 0.1% TFA. The fractions containing purified IGF were assayed by SDS-PAGE, then pooled and lyophilized.

#### Example 4

##### Recombinant Synthesis of IGFBP-3

Recombinant rhIGFBP-3 was produced using as a gene insert coding for the sequence shown in Figure 12 (SEQ ID NO:2), as detailed below. Figures 13-15 show alternative exemplary gene sequences that can be used in the present invention according to methods standard in the art or analogous to the procedures described below. Figure 13 is a preferred coding sequence used in the experiments herein.

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### Materials

The bacterial strain used in experiments carried out in support of the present invention is a derivative of Escherichia coli K-12 strain W3110 which was lysogenized with DE3. (Studier, F. and Moffat, B. [1986] J. Mol. Biol. 189: 113-130). This lysogen carries the gene for T7 RNA polymerase under the control of the lacUV5 promoter. This host strain was transformed with plasmid pDJ12833 by selection for tetracycline-resistance.

### Description of Plasmids

The three expression vectors used in this work are similar to pJU1002 and pJU1003 (Squires, C.H., et al. [1988] J. Biol. Chem. 263: 16297-16302, incorporated herein by reference) except that the gene was inserted downstream of the translational coupler IGFBP-3 (pDJ12833). In addition, pDJ12833 differs from pJU1003 in that it does not contain the synthetic 16 bp adaptor sequence at the 5' end of the tet gene in that plasmid; however, it does contain DNA insertions at the unique PvuII site in the pBR322-derived backbone: pDJ12833 contains a 385 bp fragment carrying the par locus of pSC101 (Meacock, P.A., and Cohen, S.N. [1980] Cell 20: 529-542).

### Description of Gene Insert

pDJ12833 contains an ORF comprising an ATG triplet followed by the 264 codons of mature human IGFBP-3 (Figure 13). The amino terminal 95 codons were synthetic; the remainder were derived from the natural cDNA for this gene.

In this case, the ORF was positioned relative to the translational coupler exactly as described by Squires et al. ([1988] J. Biol. Chem. 263:16297-16302,

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incorporated herein by reference) for fibroblast growth factor.

#### IGFBP-3 purification

5           The E. coli cells producing IGFBP-3 ("BP-3") were broken open using a Gaulin mill or Microfluidizer in 6 volumes/wt (e.g., 1.2 l/200 g cell paste) of 20 mM Tris-HCl and 5 mM EDTA, pH 8 while its temperature was maintained at not higher than 10°C. The lysate was  
10 checked for the presence of whole E. coli cells by light microscopy and then centrifuged at 10,000 g for 20 minutes to separate supernatant from pellet. The pellet was re-extracted twice more in 3 volumes of 20 mM Tris-HCl pH 8 and centrifuged as above. The resulting pellet  
15 was used for further purification.

          BP-3 was extracted from the pellet with 2.5 volumes/wt of 20 mM Tris pH 8.0 containing 6 M GdHCl, 25 mM DTT and 5 mM EDTA and filtered through a Sartorius 0.8  $\mu$  filter. The filtrate was diluted with the above  
20 buffer to a final protein concentration of 4 mg/ml followed with 1 volume of 20 mM Tris and 5 mM EDTA pH 8.0. This mixture was subjected to a DNA removal step using protamine sulfate, according to standard procedures known in the art. The protamine sulfate precipitate was  
25 removed by filtration, and BP-3 was subjected to the refolding reaction. A typical refolding reaction was effected at about 0.5 to 1 mg/ml protein concentration, 1.0 to 1.5 M GdHCl at pH 8-9 in the presence of Tris buffer at 20 to 50 mM, having reducing/oxidizing agents  
30 molar ratio of 1. Refolding performed as above results in greater than 60% of the initial amount of BP-3.

          The refolded BP-3 solution was clarified, buffer was exchanged using an ultrafiltration system and loaded onto a cation exchange column equilibrated in 20  
35 mM sodium phosphate buffer, pH 7. Following loading of

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the BP-3 post-refolding solution onto a cation exchange column, the column was washed with the equilibration buffer and purified BP-3 was then eluted from the column by application of 20 column volumes of gradient starting with equilibration buffer and finishing with 0.8 M sodium chloride. The appropriate fractions of BP-3 were pooled.

The salt concentration of the pooled BP-3-containing fractions was increased to 0.6 M with ammonium sulfate, and then loaded onto a hydrophobic interaction chromatography matrix column equilibrated with salt containing acetate/phosphate buffer having a pH between 5 to 6. BP-3 was eluted with a linear ammonium sulfate gradient ending with 0% ammonium sulfate buffered at pH 5-6. The appropriate fractions were then pooled and saved.

The pooled fractions containing BP-3 were brought to 0.1% trifluoroacetic acid (TFA), and the solution was loaded onto a C4 RP-HPLC column equilibrated with 0.1% TFA in water. The column was washed with equilibration buffer and then eluted with a linear gradient of acetonitrile containing 0.1% TFA. The fractions containing BP-3 were pooled and lyophilized.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: CELTRIX PHARMACEUTICALS, INC.
- (ii) TITLE OF INVENTION: IGF/IGFBP COMPLEX FOR PROMOTING BONE FORMATION AND FOR REGULATING BONE REMODELING
- (iii) NUMBER OF SEQUENCES: 7
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: MORRISON & FOERSTER
  - (B) STREET: 755 Page Mill Road
  - (C) CITY: Palo Alto
  - (D) STATE: California
  - (E) COUNTRY: USA
  - (F) ZIP: 94304-1018
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: Patent In Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: New
  - (B) FILING DATE: New
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: PARK, FREDDIE K.
  - (B) REGISTRATION NUMBER: 35,636
  - (C) REFERENCE/DOCKET NUMBER: 2095-2072.40
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (415) 813-5600
  - (B) TELEFAX: (415) 494-0792
  - (C) TELEX: 706141

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 70 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:  

Gly	Pro	Glu	Thr	Leu	Cys	Gly	Ala	Glu	Leu	Val	Asp	Ala	Leu	Gln	Phe
1				5				10						15	
Val	Cys	Gly	Asp	Arg	Gly	Phe	Tyr	Phe	Asn	Lys	Pro	Thr	Gly	Tyr	Gly
			20					25					30		
Ser	Ser	Ser	Arg	Arg	Ala	Pro	Gln	Thr	Gly	Ile	Val	Asp	Glu	Cys	Cys
			35				40					45			
Phe	Arg	Ser	Cys	Asp	Leu	Arg	Arg	Leu	Glu	Met	Tyr	Cys	Ala	Pro	Leu
			50			55				60					

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Lys Pro Ala Lys Ser Ala  
65 70

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 264 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 5
- (D) OTHER INFORMATION: /note= "There is natural heterogeneity at this position; glycine can also occur at this position."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly	Ala	Ser	Ser	Ala	Gly	Leu	Gly	Pro	Val	Val	Arg	Cys	Glu	Pro	Cys	
1				5				10						15		
Asp	Ala	Arg	Ala	Leu	Ala	Gln	Cys	Ala	Pro	Pro	Pro	Ala	Val	Cys	Ala	
			20				25						30			
Glu	Leu	Val	Arg	Glu	Pro	Gly	Cys	Gly	Cys	Cys	Leu	Thr	Cys	Ala	Leu	
		35				40					45					
Ser	Glu	Gly	Gln	Pro	Cys	Gly	Ile	Tyr	Thr	Glu	Arg	Cys	Gly	Ser	Gly	
	50					55					60					
Leu	Arg	Cys	Gln	Pro	Ser	Pro	Asp	Glu	Ala	Arg	Pro	Leu	Gln	Ala	Leu	
65				70					75						80	
Leu	Asp	Gly	Arg	Gly	Leu	Cys	Val	Asn	Ala	Ser	Ala	Val	Ser	Arg	Leu	
			85					90						95		
Arg	Ala	Tyr	Leu	Pro	Ala	Pro	Pro	Ala	Pro	Gly	Asn	Ala	Ser	Glu		
			100			105						110				
Ser	Glu	Glu	Asp	Arg	Ser	Ala	Gly	Ser	Val	Glu	Ser	Pro	Ser	Val	Ser	
		115				120						125				
Ser	Thr	His	Arg	Val	Ser	Asp	Pro	Lys	Phe	His	Pro	Leu	His	Ser	Lys	
		130				135					140					
Ile	Ile	Ile	Ile	Lys	Lys	Gly	His	Ala	Lys	Asp	Ser	Gln	Arg	Tyr	Lys	
145				150						155					160	
Val	Asp	Tyr	Glu	Ser	Gln	Ser	Thr	Asp	Thr	Gln	Asn	Phe	Ser	Ser	Glu	
			165						170					175		
Ser	Lys	Arg	Glu	Thr	Glu	Tyr	Gly	Pro	Cys	Arg	Arg	Glu	Met	Glu	Asp	
			180					185					190			
Thr	Leu	Asn	His	Leu	Lys	Phe	Leu	Asn	Val	Leu	Ser	Pro	Arg	Gly	Val	
		195				200						205				
His	Ile	Pro	Asn	Cys	Asp	Lys	Lys	Gly	Phe	Tyr	Lys	Lys	Lys	Gln	Cys	
	210					215					220					
Arg	Pro	Ser	Lys	Gly	Arg	Lys	Arg	Gly	Phe	Cys	Trp	Cys	Val	Asp	Lys	
225				230						235					240	

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Tyr Gly Gln Pro Leu Pro Gly Tyr Thr Thr Lys Gly Lys Glu Asp Val  
 245 250 255

His Cys Tyr Ser Met Gln Ser Lys  
 260

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 795 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ix) FEATURE:

- (A) NAME/KEY: CDS  
 (B) LOCATION: 1..792

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATG GGT GCA TCT TCT GCA GGT TTA GGT CCA GTT GTT CGT TGT GAA CCA	48
Met Gly Ala Ser Ser Ala Gly Leu Gly Pro Val Val Arg Cys Glu Pro	
1 5 10 15	
TGT GAT GCT CGT GCT CTT GCT CAA TGT GCT CCA CCA GCT GTT TGT GCT	96
Cys Asp Ala Arg Ala Leu Ala Gln Cys Ala Pro Pro Ala Val Cys Ala	
20 25 30	
GAA CTT GTT CGT GAA CCG GGT TGT GGT TGT TGT CTG ACT TGC GCA CTT	144
Glu Leu Val Arg Glu Pro Gly Cys Gly Cys Cys Leu Thr Cys Ala Leu	
35 40 45	
TCT GAA GGT CAA CCA TGT GGT ATT TAT ACT GAA CGT TGT GGT TCT GGT	192
Ser Glu Gly Gln Pro Cys Gly Ile Tyr Thr Glu Arg Cys Gly Ser Gly	
50 55 60	
CTG CGT TGT CAA CCA TCT CCA GAT GAA GCT CGT CCT CTG CAG GCT CTG	240
Leu Arg Cys Gln Pro Ser Pro Asp Glu Ala Arg Pro Leu Gln Ala Leu	
65 70 75 80	
CTG GAC GGT CGT GGT CTG TGC GTT AAC GCT TCC GCT GTT TCC CGT CTG	288
Leu Asp Gly Arg Gly Leu Cys Val Asn Ala Ser Ala Val Ser Arg Leu	
85 90 95	
CGC GCC TAC CTG CTG CCA GCG CCG CCA GCT CCA GGA AAT GCT AGT GAG	336
Arg Ala Tyr Leu Leu Pro Ala Pro Pro Ala Pro Gly Asn Ala Ser Glu	
100 105 110	
TCG GAG GAA GAC CGC AGC GCC GGC AGT GTG GAG AGC CCG TCC GTC TCC	384
Ser Glu Glu Asp Arg Ser Ala Gly Ser Val Glu Ser Pro Ser Val Ser	
115 120 125	
AGC ACG CAC CGG GTG TCT GAT CCC AAG TTC CAC CCC CTC CAT TCA AAG	432
Ser Thr His Arg Val Ser Asp Pro Lys Phe His Pro Leu His Ser Lys	
130 135 140	
ATA ATC ATC ATC AAG AAA GGG CAT GCT AAA GAC AGC CAG CGC TAC AAA	480
Ile Ile Ile Ile Lys Lys Gly His Ala Lys Asp Ser Gln Arg Tyr Lys	
145 150 155 160	
GTT GAC TAC GAG TCT CAG AGC ACA GAT ACC CAG AAC TTC TCC TCC GAG	528
Val Asp Tyr Glu Ser Gln Ser Thr Asp Thr Gln Asn Phe Ser Ser Glu	
165 170 175	

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TCC AAG CGG GAG ACA GAA TAT GGT CCC TGC CGT AGA GAA ATG GAA GAC Ser Lys Arg Glu Thr Glu Tyr Gly Pro Cys Arg Arg Glu Met Glu Asp 180 185 190	576
ACA CTG AAT CAC CTG AAG TTC CTC AAT GTG CTG AGT CCC AGG GGT GTA Thr Leu Asn His Leu Lys Phe Leu Asn Val Leu Ser Pro Arg Gly Val 195 200 205	624
CAC ATT CCC AAC TGT GAC AAG AAG GGA TTT TAT AAG AAA AAG CAG TGT His Ile Pro Asn Cys Asp Lys Lys Gly Phe Tyr Lys Lys Lys Gln Cys 210 215 220	672
CGC CCT TCC AAA GGC AGG AAG CGG GGC TTC TGC TGG TGT GTG GAT AAG Arg Pro Ser Lys Gly Arg Lys Arg Gly Phe Cys Trp Cys Val Asp Lys 225 230 235 240	720
TAT GGG CAG CCT CTG CCA GGC TAC ACC ACC AAG GGG AAG GAG GAC GTG Tyr Gly Gln Pro Leu Pro Gly Tyr Thr Thr Lys Gly Lys Glu Asp Val 245 250 255	768
CAC TGC TAC AGC ATG CAG AGC AAG TAG His Cys Tyr Ser Met Gln Ser Lys 260	795

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 264 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Gly Ala Ser Ser Ala Gly Leu Gly Pro Val Val Arg Cys Glu Pro 1 5 10 15
Cys Asp Ala Arg Ala Leu Ala Gln Cys Ala Pro Pro Ala Val Cys Ala 20 25 30
Glu Leu Val Arg Glu Pro Gly Cys Gly Cys Cys Leu Thr Cys Ala Leu 35 40 45
Ser Glu Gly Gln Pro Cys Gly Ile Tyr Thr Glu Arg Cys Gly Ser Gly 50 55 60
Leu Arg Cys Gln Pro Ser Pro Asp Glu Ala Arg Pro Leu Gln Ala Leu 65 70 75 80
Leu Asp Gly Arg Gly Leu Cys Val Asn Ala Ser Ala Val Ser Arg Leu 85 90 95
Arg Ala Tyr Leu Leu Pro Ala Pro Pro Ala Pro Gly Asn Ala Ser Glu 100 105 110
Ser Glu Glu Asp Arg Ser Ala Gly Ser Val Glu Ser Pro Ser Val Ser 115 120 125
Ser Thr His Arg Val Ser Asp Pro Lys Phe His Pro Leu His Ser Lys 130 135 140
Ile Ile Ile Ile Lys Lys Gly His Ala Lys Asp Ser Gln Arg Tyr Lys 145 150 155 160

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[illegible]

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 811 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGTGCTTCTT	CTGCTGGTCT	TGGACCAAGTT	GTTTCGTTGTG	AACCATGTGA	TGCACGAGCT	60
TTAGCTCAAT	GTGCTCCACC	ACCAGCTGTT	TGTGCTGAAT	TAGTTCGAGA	ACCAGGTTGT	120
GGTTGTTGTT	TAACTTGTGC	TTTATCTGAA	GGTCAACCAT	GTGGTATTTA	TACTGAACGT	180
TGCGGTAGTG	GTTTGCCTTG	TCAACCAAGC	CCAGATGAAG	CTAGGCCTTT	ACAAGCATT	240
TTAGATGGTC	GAGGTCTGTG	TGTTAATGCG	TCCGCTGTTT	CTCGATTGCG	CGCTTATTTA	300
TTACCTGCCC	CACCGGCACC	GGGTAACGCC	TCCGAAAGCG	AAGAGGATCG	TTCTGCGGGT	360
TCCGTTGAAT	CTCCAAGTGT	GAGTTCCTACC	CATCGAGTTA	GCGACCCGAA	ATTTTCATCCG	420
TTGCACTCTA	AAATCATTAT	TATTA AAAAG	GGTCACGCAA	AGGATTCTCA	ACGTTATAAG	480
GTGGATTATG	AAAGCCAATC	TACCGACACT	CAAAATTTTA	GTAGTGAAAG	TAAACGTGAA	540
ACCGAGTACG	GCCCGTGTCG	ACGTGAGATG	GAGGATACCT	TAAACCATT	AAAATTTTGT	600
AACGTTTTAT	CCCCGCGTGG	CGTTCATATC	CCGAATTGCG	ATAAAAAAGG	CTTCTACAAA	660
AAGAAACAAT	GCCGTCCGAG	TAAGGGTCGT	AAACGAGGTT	TTTGTGGTG	CGTTGACAAA	720
TACGGTCAAC	CGTTGCCGGG	TTATACTACT	AAAGGCAAAG	AAGATGTTCA	TTGTTATTCT	780
ATGCAATCTA	AATAATGCAT	CTCGAGAATT	C			811

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 876 base pairs



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(B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ix) FEATURE:

(A) NAME/KEY: CDS  
 (B) LOCATION: 1..873

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATG CAG CGG GCG CGA CCC ACG CTC TGG GCC GCT GCG CTG ACT CTG CTG	48
Met Gln Arg Ala Arg Pro Thr Leu Trp Ala Ala Ala Leu Thr Leu Leu	
265 270 275 280	
GTG CTG CTC CGC GGG CCG CCG GTG GCG CGG GCT GGC GCG AGC TCG GCG	96
Val Leu Leu Arg Gly Pro Pro Val Ala Arg Ala Gly Ala Ser Ser Ala	
285 290 295	
GGC TTG GGT CCC GTG GTG CGC TGC GAG CCG TGC GAC GCG CGT GCA CTG	144
Gly Leu Gly Pro Val Val Arg Cys Glu Pro Cys Asp Ala Arg Ala Leu	
300 305 310	
GCC CAG TGC GCG CCT CCG CCC GCC GTG TGC GCG GAG CTG GTG CGC GAG	192
Ala Gln Cys Ala Pro Pro Pro Ala Val Cys Ala Glu Leu Val Arg Glu	
315 320 325	
CCG GGC TGC GGC TGC TGC CTG ACG TGC GCA CTG AGC GAG GGC CAG CCG	240
Pro Gly Cys Gly Cys Cys Leu Thr Cys Ala Leu Ser Glu Gly Gln Pro	
330 335 340	
TGC GGC ATC TAC ACC GAG CGC TGT GGC TCC GGC CTT CGC TGC CAG CCG	288
Cys Gly Ile Tyr Thr Glu Arg Cys Gly Ser Gly Leu Arg Cys Gln Pro	
345 350 355 360	
TCG CCC GAC GAG GCG CGA CCG CTG CAG GCG CTG CTG GAC GGC CGC GGG	336
Ser Pro Asp Glu Ala Arg Pro Leu Gln Ala Leu Leu Asp Gly Arg Gly	
365 370 375	
CTC TGC GTC AAC GCT AGT GCC GTC AGC CGC CTG CGC GCC TAC CTG CTG	384
Leu Cys Val Asn Ala Ser Ala Val Ser Arg Leu Arg Ala Tyr Leu Leu	
380 385 390	
CCA GCG CCG CCA GCT CCA GGA AAT GCT AGT GAG TCG GAG GAA GAC CGC	432
Pro Ala Pro Pro Ala Pro Gly Asn Ala Ser Glu Ser Glu Glu Asp Arg	
395 400 405	
AGC GCC GGC AGT GTG GAG AGC CCG TCC GTC TCC AGC ACG CAC CGG GTG	480
Ser Ala Gly Ser Val Glu Ser Pro Ser Val Ser Ser Thr His Arg Val	
410 415 420	
TCT GAT CCC AAG TTC CAC CCC CTC CAT TCA AAG ATA ATC ATC ATC AAG	528
Ser Asp Pro Lys Phe His Pro Leu His Ser Lys Ile Ile Ile Ile Lys	
425 430 435 440	
AAA GGG CAT GCT AAA GAC AGC CAG CGC TAC AAA GTT GAC TAC GAG TCT	576
Lys Gly His Ala Lys Asp Ser Gln Arg Tyr Lys Val Asp Tyr Glu Ser	
445 450 455	
CAG AGC ACA GAT ACC CAG AAC TTC TCC TCC GAG TCC AAG CGG GAG ACA	624
Gln Ser Thr Asp Thr Gln Asn Phe Ser Ser Glu Ser Lys Arg Glu Thr	
460 465 470	
GAA TAT GGT CCC TGC CGT AGA GAA ATG GAA GAC ACA CTG AAT CAC CTG	672
Glu Tyr Gly Pro Cys Arg Arg Glu Met Glu Asp Thr Leu Asn His Leu	
475 480 485	

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AAG TTC CTC AAT GTG CTG AGT CCC AGG GGT GTA CAC ATT CCC AAC TGT Lys Phe Leu Asn Val Leu Ser Pro Arg Gly Val His Ile Pro Asn Cys 490 495 500	720
GAC AAG AAG GGA TTT TAT AAG AAA AAG CAG TGT CGC CCT TCC AAA GGC Asp Lys Lys Gly Phe Tyr Lys Lys Lys Gln Cys Arg Pro Ser Lys Gly 505 510 515 520	768
AGG AAG CGG GGC TTC TGC TGG TGT GTG GAT AAG TAT GGG CAG CCT CTC Arg Lys Arg Gly Phe Cys Trp Cys Val Asp Lys Tyr Gly Gln Pro Leu 525 530 535	816
CCA GGC TAC ACC ACC AAG GGG AAG GAG GAC GTG CAC TGC TAC AGC ATG Pro Gly Tyr Thr Thr Lys Gly Lys Glu Asp Val His Cys Tyr Ser Met 540 545 550	864
CAG AGC AAG TAG Gln Ser Lys 555	876

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 291 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Gln Arg Ala Arg Pro Thr Leu Trp Ala Ala Ala Leu Thr Leu Leu 1 5 10 15
Val Leu Leu Arg Gly Pro Pro Val Ala Arg Ala Gly Ala Ser Ser Ala 20 25 30
Gly Leu Gly Pro Val Val Arg Cys Glu Pro Cys Asp Ala Arg Ala Leu 35 40 45
Ala Gln Cys Ala Pro Pro Pro Ala Val Cys Ala Glu Leu Val Arg Glu 50 55 60
Pro Gly Cys Gly Cys Cys Leu Thr Cys Ala Leu Ser Glu Gly Gln Pro 65 70 75 80
Cys Gly Ile Tyr Thr Glu Arg Cys Gly Ser Gly Leu Arg Cys Gln Pro 85 90 95
Ser Pro Asp Glu Ala Arg Pro Leu Gln Ala Leu Leu Asp Gly Arg Gly 100 105 110
Leu Cys Val Asn Ala Ser Ala Val Ser Arg Leu Arg Ala Tyr Leu Leu 115 120 125
Pro Ala Pro Pro Ala Pro Gly Asn Ala Ser Glu Ser Glu Glu Asp Arg 130 135 140
Ser Ala Gly Ser Val Glu Ser Pro Ser Val Ser Ser Thr His Arg Val 145 150 155 160
Ser Asp Pro Lys Phe His Pro Leu His Ser Lys Ile Ile Ile Ile Lys 165 170 175

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Lys Gly His Ala Lys Asp Ser Gln Arg Tyr Lys Val Asp Tyr Glu Ser  
180 185 190  
Gln Ser Thr Asp Thr Gln Asn Phe Ser Ser Glu Ser Lys Arg Glu Thr  
195 200 205  
Glu Tyr Gly Pro Cys Arg Arg Glu Met Glu Asp Thr Leu Asn His Leu  
210 215 220  
Lys Phe Leu Asn Val Leu Ser Pro Arg Gly Val His Ile Pro Asn Cys  
225 230 235 240  
Asp Lys Lys Gly Phe Tyr Lys Lys Lys Gln Cys Arg Pro Ser Lys Gly  
245 250 255  
Arg Lys Arg Gly Phe Cys Trp Cys Val Asp Lys Tyr Gly Gln Pro Leu  
260 265 270  
Pro Gly Tyr Thr Thr Lys Gly Lys Glu Asp Val His Cys Tyr Ser Met  
275 280 285  
Gln Ser Lys  
290

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## CLAIMS

1. A method for stimulating bone formation in  
a subject who has a bone marrow disorder causing bone  
5 loss, said method comprising administering to the subject  
pharmaceutically effective doses of IGF-I and IGFBP-3.
2. The method of claim 1 wherein the marrow  
disorder is selected from the group consisting of plasma  
10 cell dyscrasias, leukemia, lymphomas, systemic  
mastocytosis, anemias, lipidoses and mucopoly-  
saccharidoses.
3. The method of claim 1 wherein a  
15 pharmaceutically effective dose of an inhibitor of bone  
resorption is also administered.
4. The method of claim 3, wherein the IGF-I/  
IGFBP and the inhibitor of bone resorption are  
20 administered sequentially.
5. The method of claim 3, wherein the  
inhibitor of bone resorption is an estrogen, tamoxifen, a  
calcitonin, a bisphosphonate or a growth factor having  
25 anti-resorptive activity.
6. The method of claim 5 wherein the growth  
factor is a transforming growth factor- $\beta$ .
7. The method of claim 3, wherein the  
30 treatment is prophylactic.
8. A method for stimulating bone formation in  
a subject who has a connective tissue disorder causing  
35 bone loss, said method comprising administering to the

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subject pharmaceutically effective doses of IGF-I and IGFBP-3.

9. The method of claim 8 wherein the  
5 connective tissue disorder is selected from the group consisting of osteogenesis imperfecta, Ehlers-Danlos syndrome, Marfans syndrome, cutis laxa, homocystinuria, Mankes's syndrome and scurvy.

10 10. The method of claim 9 wherein a pharmaceutically effective dose of an inhibitor of bone resorption is also administered.

15 11. A method for stimulating bone formation in a subject who has drug-related osteoporosis, said method comprising administering to the subject pharmaceutically effective doses of IGF-I and IGFBP-3.

20 12. The method of claim 11 wherein the drug causing the osteoporosis is selected from the group consisting of corticosteroids, heparin, oral anticoagulants, anticonvulsants, methotrexate, thyroid hormone, lithium and gonadotrophin-releasing analogs.

25 13. The method of claim 11 wherein a pharmaceutically effective dose of an inhibitor of bone resorption is also administered.

30 14. A method for stimulating bone formation in a subject who has bone loss due to pregnancy, lactation, chronic hypophosphatemia, hyperphosphatasia, insulin-dependent diabetes mellitus, anorexia nervosa, cadmium poisoning, juvenile osteoporosis or Paget's disease of bone, said method comprising administering to the subject  
35 pharmaceutically effective doses of IGF-I and IGFBP-3.

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15. The method of claim 14 wherein a pharmaceutically effective dose of an inhibitor of bone resorption is also administered.

5           16. A method for stimulating bone formation in a subject who has periodontal bone loss, said method comprising administering to the subject pharmaceutically effective doses of IGF-I and IGFBP-3.

10           17. A method for stimulating bone formation in a subject who has osteoarthritis-related bone loss, said method comprising administering to the subject pharmaceutically effective doses of IGF-I and IGFBP-3.

15           18. A composition for inducing bone formation in a subject, comprising IGF-I, IGFBP and an inhibitor of bone resorption in a pharmaceutically acceptable excipient.

20           19. The composition of claim 18, wherein the inhibitor of bone resorption is an estrogen, tamoxifen, a calcitonin, a bisphosphonate or a growth factor having anti-resorptive activity.

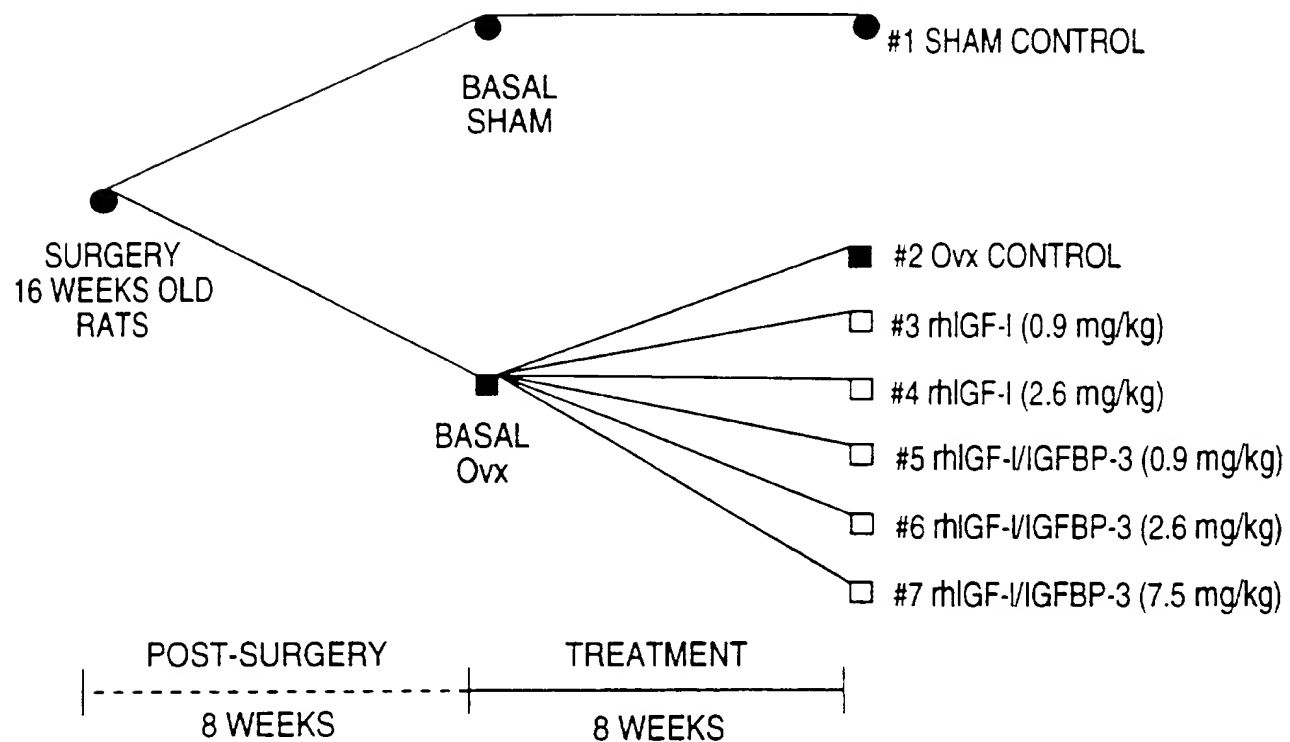
25           20. The composition of claim 18, wherein the IGF-I or the IGFBP is conjugated to the inhibitor of bone resorption.

30           21. The composition of claim 18, further comprising a sustained-release vehicle.

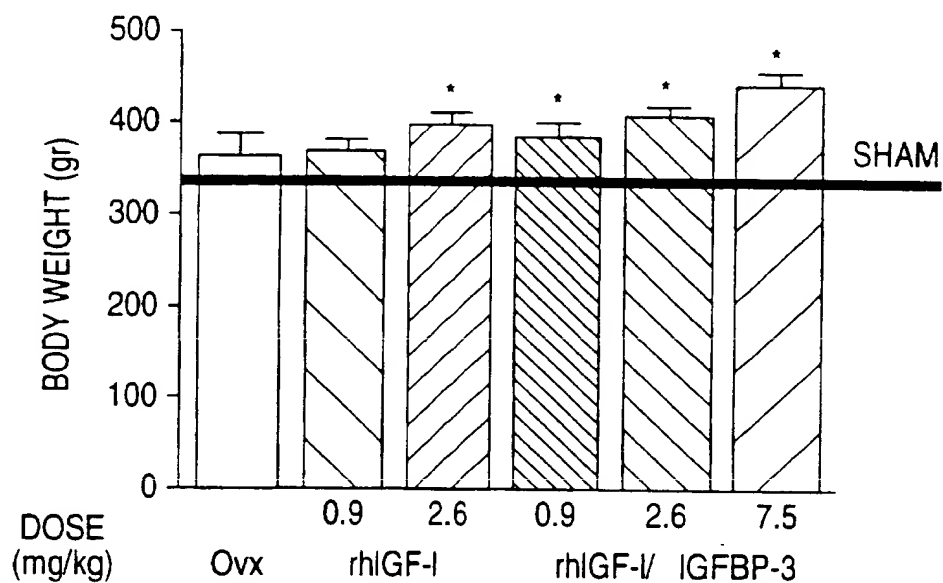
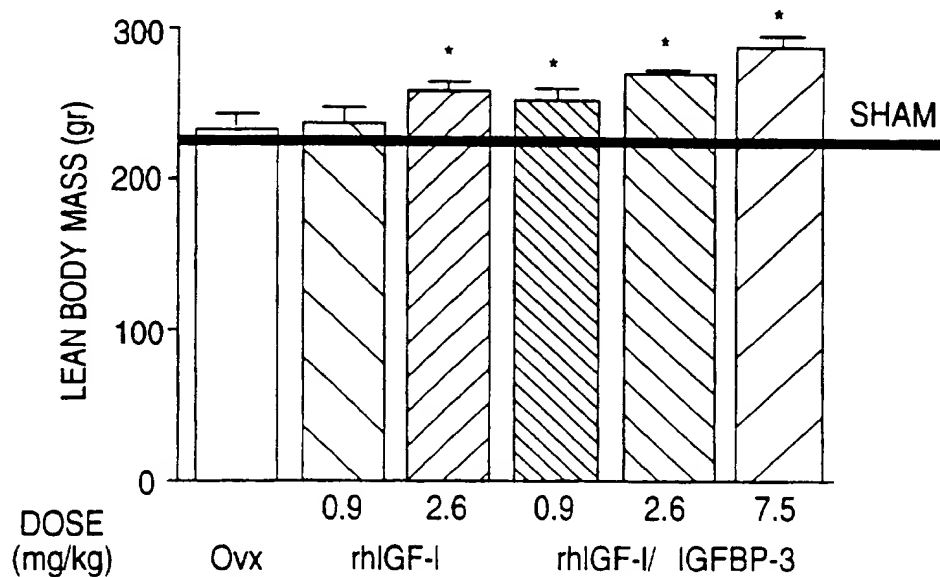
            22. The composition of claim 18, wherein the IGFBP is IGFBP-3.

35

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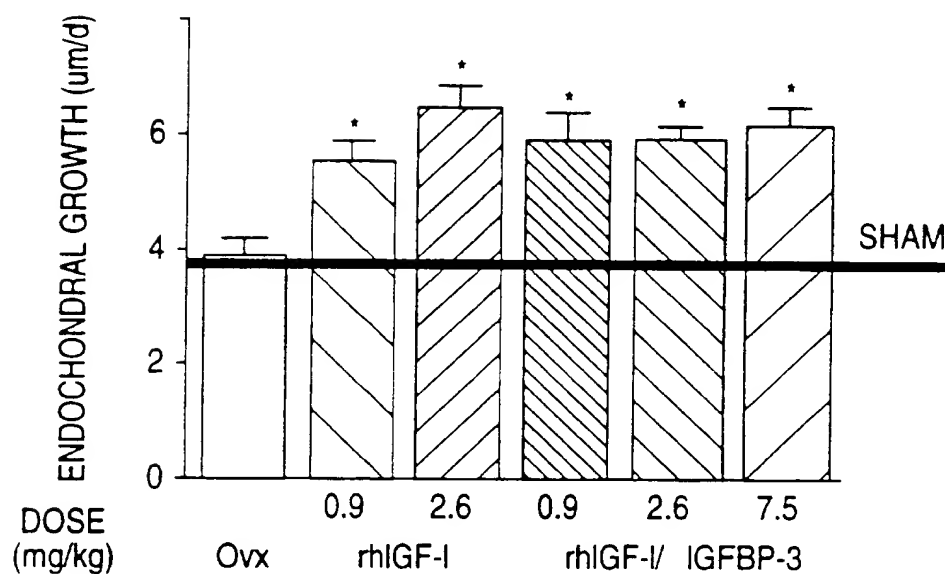
**FIG. 1**

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**FIG. 2A**\*SIGNIFICANTLY DIFFERENT  
FROM Ovex GROUP**FIG. 2B**\*SIGNIFICANTLY DIFFERENT  
FROM Ovex GROUP



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**FIG. 3**

\*SIGNIFICANTLY DIFFERENT  
FROM Ovex GROUP

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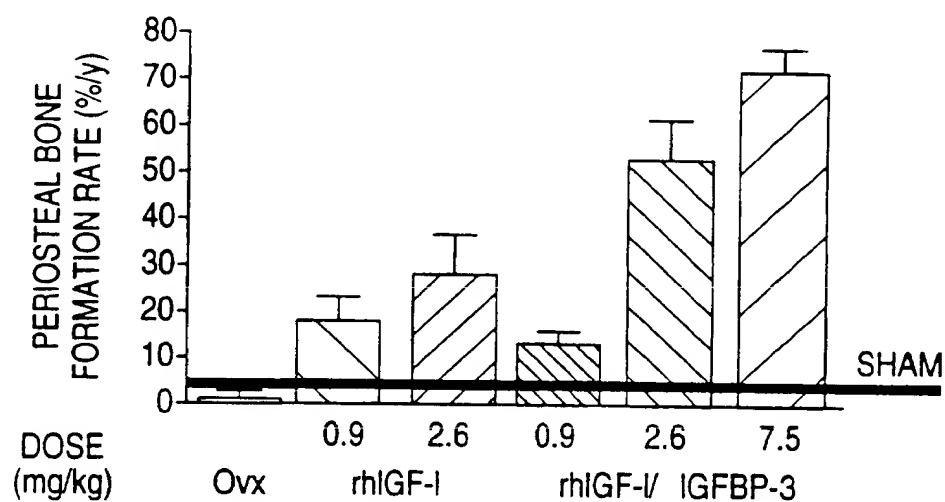


FIG. 4A

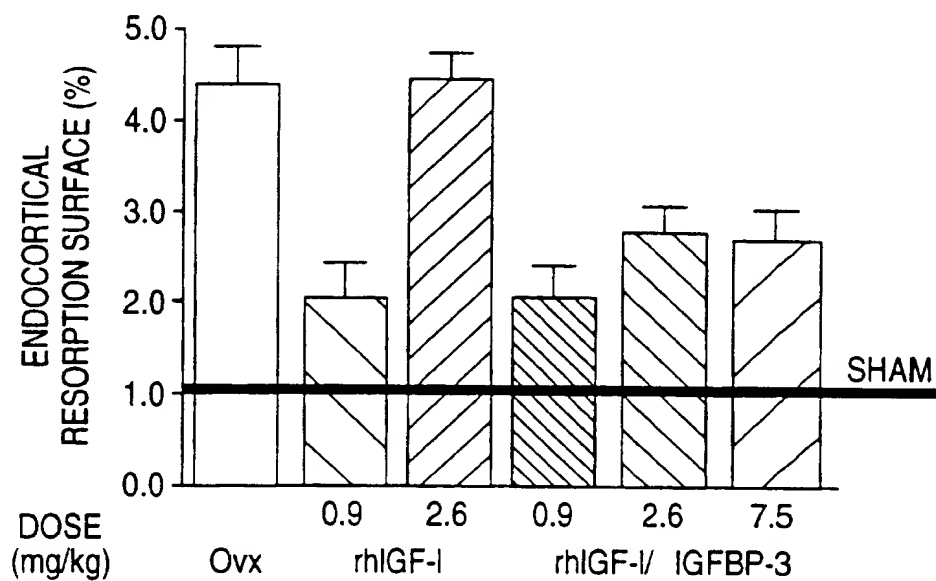


FIG. 4B

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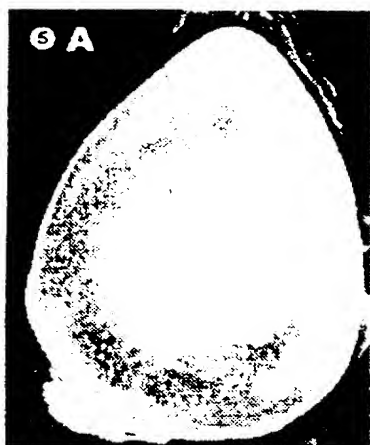


FIG. 5A



FIG. 5B

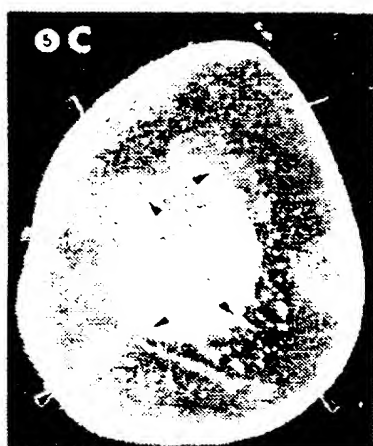


FIG. 5C



FIG. 5D

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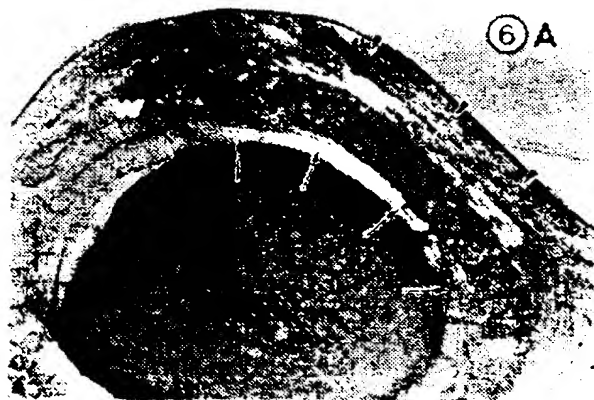


FIG. 6A

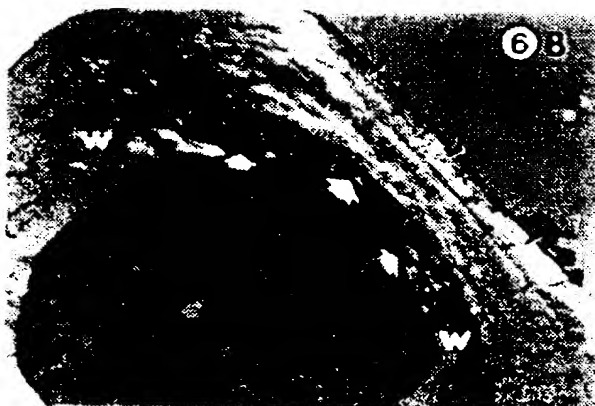


FIG. 6B

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FIG. 6C



FIG. 6D

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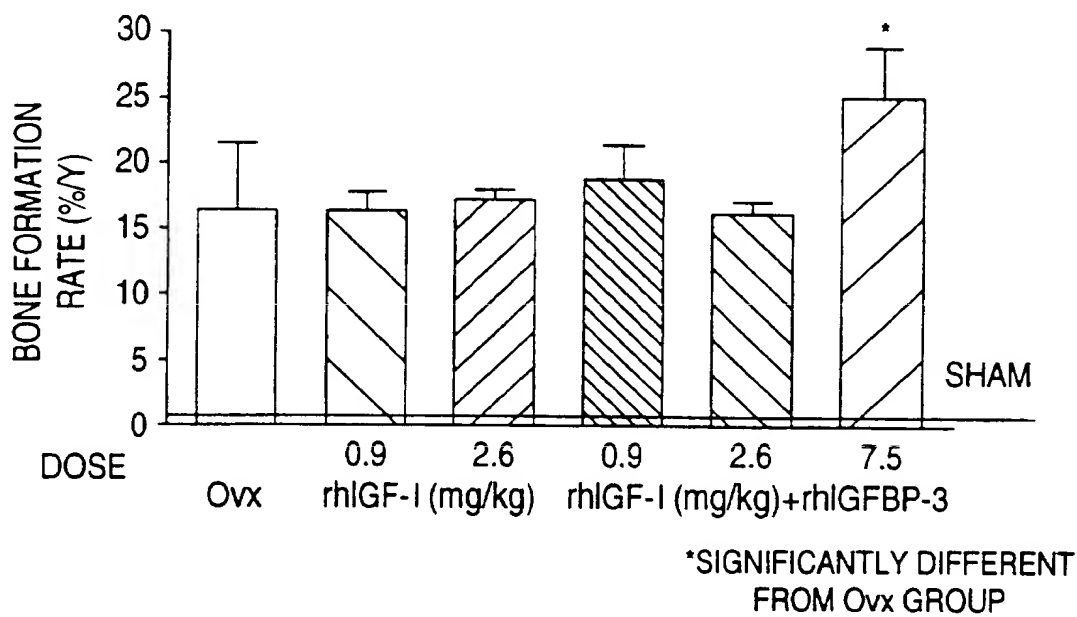


FIG. 7A

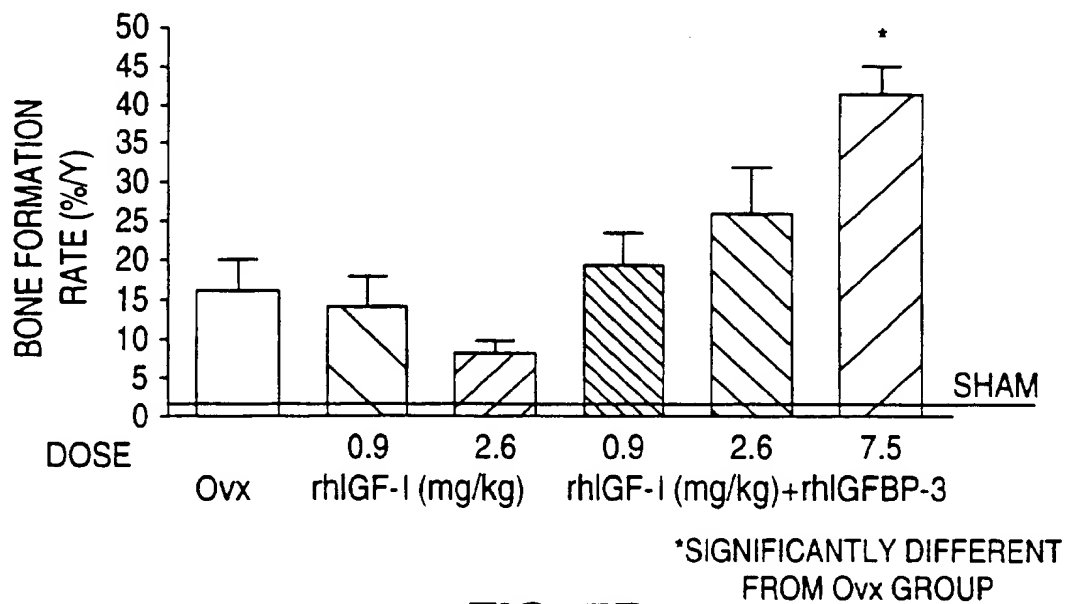
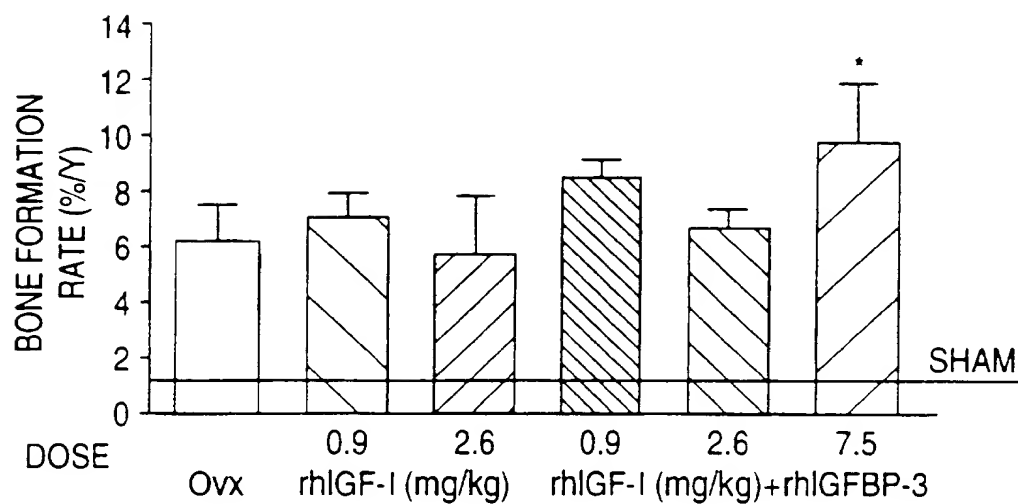
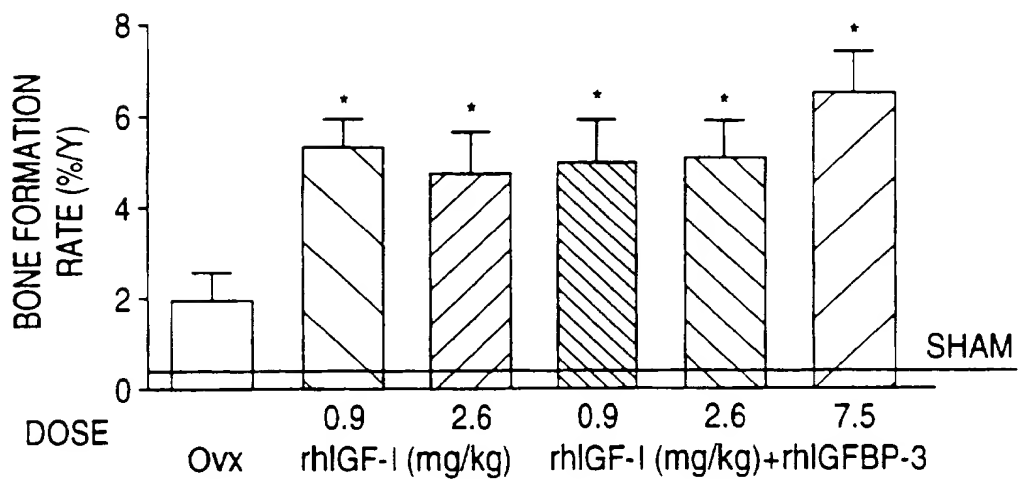
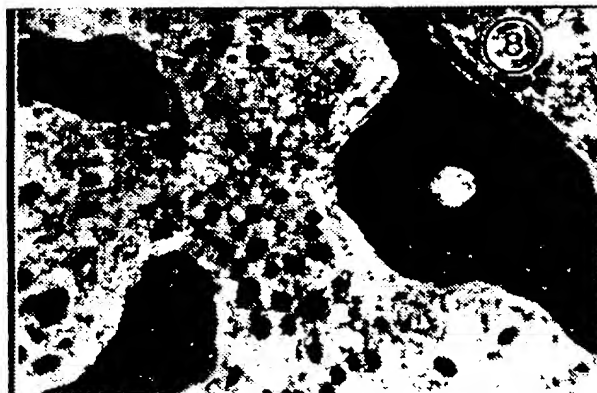


FIG. 7B

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**FIG. 7C**\*SIGNIFICANTLY DIFFERENT  
FROM OvX GROUP**FIG. 7D**\*SIGNIFICANTLY DIFFERENT  
FROM OvX GROUP

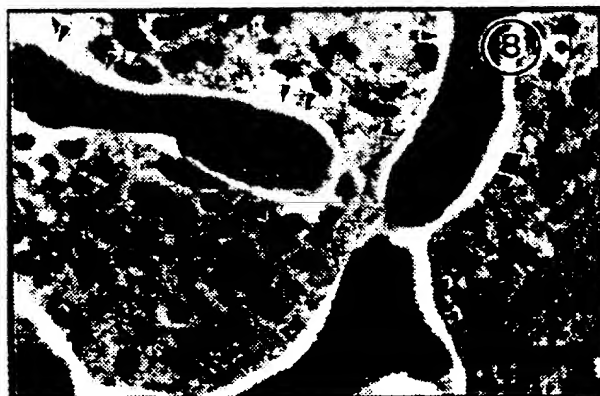
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**FIG. 8A**



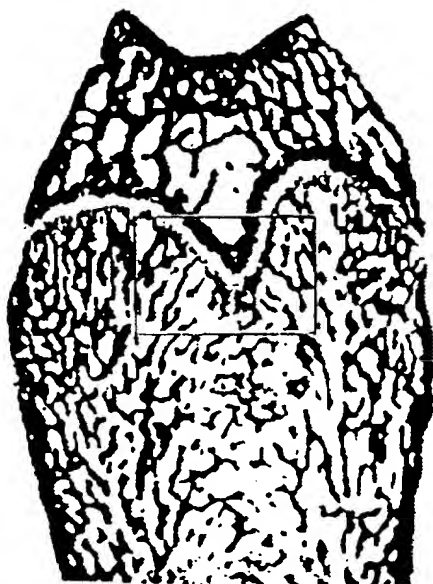
**FIG. 8B**



**FIG. 8C**



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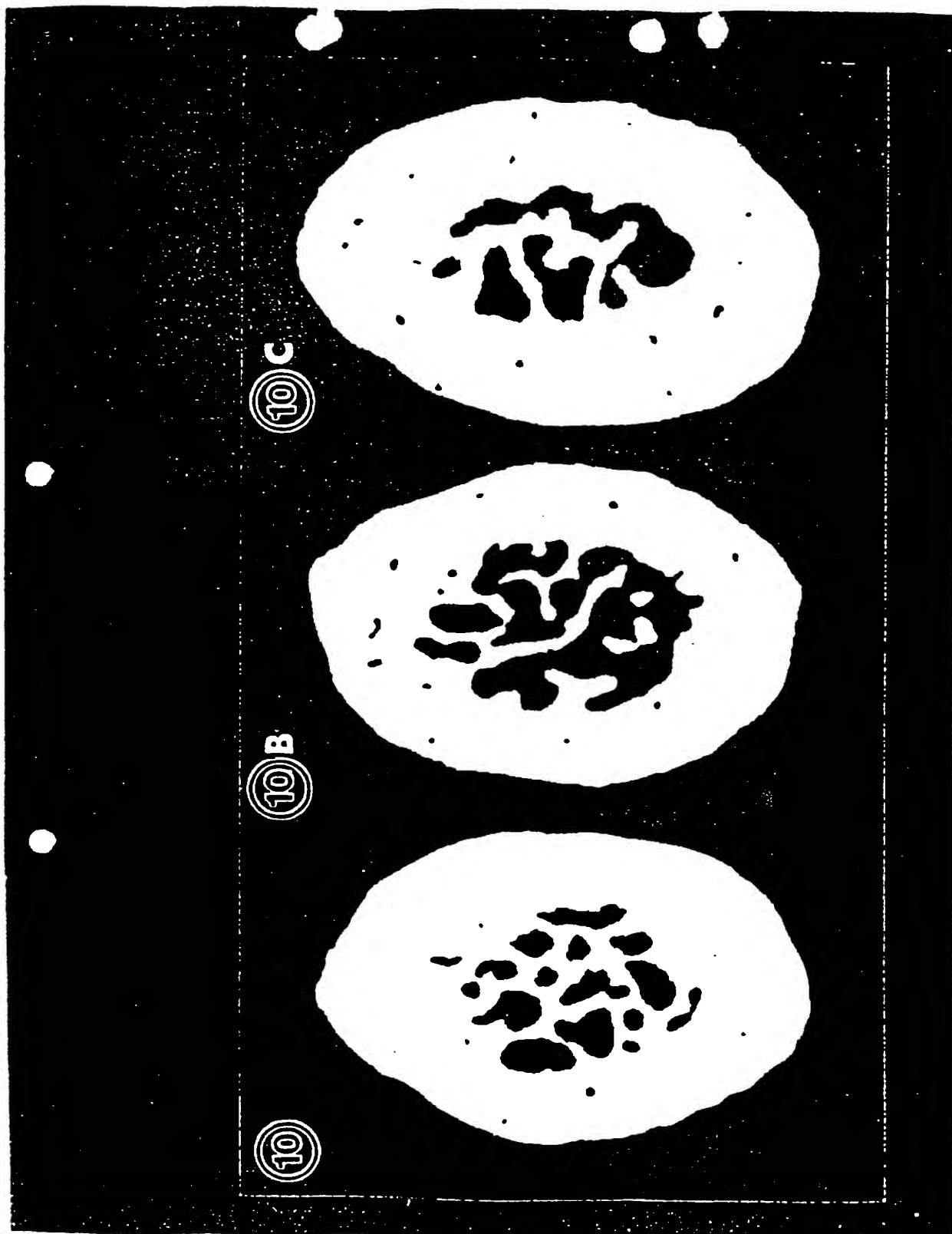
**FIG. 9A**



**FIG. 9B**



**FIG. 9C**



F 10A 10B 10C

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GPETLCGAELVDALQFVCGDRGFYFNK  
PTGYGSSSRRAPQTGIVDECCFRSCDL  
RRLEMYCAPLKPAKSA

**FIG. 11**

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\*

GASSAGLGPVVRCEPCDARALAQCAPP PAVCAELVRE  
PGCGCCLTCA LSEGQPCGIYTERCGSGLRCQPSPDEA  
RPLQALLDGRGLCVNASAVSRLRAYLLPAPPAPGNAS  
ESEEDRSAGSVESPSVSSTHRVSDPKFHPLHSKIIII  
KKGHAKDSQRYKVDYESQSTDTONFSSESKRETEYGP  
CRREMEDTLNHLKFLNVLSPRGVHIPNCDKKGFYKKK  
QCRPSKGRKRGFCWCVDKYGQPLPGYTTKGKEDVHCY  
SMQSK

\* There is a natural heterogeneity at position 5; glycine can also occur at this position.

**FIG. 12**

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```

156  ATG GGT GCA TCT TCT GCA GGT TTA GGT CCA GTT GTT CGT TGT GAA CCA
      Met Gly Ala Ser Ser 5      Ala Gly Leu Gly Pro Val Val Arg Cys Glu Pro
      1                                10      15

204  TGT GAT GCT CGT GCT CTT GCT CAA TGT GCT CCA CCA CCA GCT GTT TGT
      Cys Asp Ala Arg Ala Leu Ala Gln Cys Ala Pro Pro Ala Val Cys
      20      25      30

252  GCT GAA CTT GTT CGT GAA CCG GGT TGT GGT TGT CTG ACT TGC GCA
      Ala Glu Leu Leu Val Arg Glu Pro Gly Cys Gly Cys Cys Leu Thr Cys Ala
      35      40      45

300  CTT TCT GAA GGT CAA CCA TGT GGT ATT TAT ACT GAA CGT TGT GGT TCT
      Leu Ser Glu Gly Gln Pro Cys Gly Ile Tyr Thr Glu Arg Cys Gly Ser
      50      55      60

348  GGT CTG CGT TGT CAA CCA TCT CCA GAT GAA GCT CGT CCT CTG CAG GCT
      Gly Leu Arg Cys Gln Pro Ser Pro Asp Glu Ala Arg Pro Leu Gln Ala
      65      70      75      80

```

FIG. 13A

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```

396  CTG CTG GAC GGT CGT GGT CTG TGC GTT AAC GCT TCC GCT GTT TCC CGT
      Leu Leu Asp Gly Arg Gly Leu Cys Val Asn Ala Ser Ala Val Ser Arg
      85                                     90                                     95

444  CTG CGC GCC TAC CTG CTG CCA GCG CCG CCA GCT CCA GGA AAT GCT AGT
      Leu Arg Ala Tyr Leu Leu Pro Ala Pro Pro Ala Pro Gly Asn Ala Ser
      100                                     105                                     110

492  GAG TCG GAG GAA GAC CGC AGC GCC GGC AGT GTG GAG AGC CCG TCC GTC
      Glu Ser Glu Glu Asp Arg Ser Ala Gly Ser Val Glu Ser Pro Ser Val
      115                                     120                                     125

540  TCC AGC ACG CAC CGG GTG TCT GAT CCC AAG TTC CAC CCC CTC CAT TCA
      Ser Ser Thr His Arg Val Ser Asp Pro Lys Phe His Pro Leu His Ser
      130                                     135                                     140

588  AAG ATA ATC ATC ATC AAG AAA GGG CAT GCT AAA GAC AGC CAG CGC TAC
      Lys Ile Ile Ile Ile Lys Lys Gly His Ala Lys Asp Ser Gln Arg Tyr
      145                                     150                                     155                                     160

```

FIG. 13B

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636	AAA GTT GAC TAC GAG TCT CAG AGC ACA GAT ACC CAG AAC TTC TCC TCC	175
	Lys Val Asp Tyr Glu Ser Gln Ser Thr Asp Thr Gln Asn Phe Ser Ser	
		165
684	GAG TCC AAG CGG GAG ACA GAA TAT GGT CCC TGC CGT AGA GAA ATG GAA	190
	Glu Ser Lys Arg Glu Thr Glu Tyr Gly Pro Cys Arg Arg Glu Met Glu	
		180
		185
732	GAC ACA CTG AAT CAC CTG AAG TTC CTC AAT GTG CTG AGT CCC AGG GGT	205
	Asp Thr Leu Asn His Leu Lys Phe Leu Asn Val Leu Ser Pro Arg Gly	
		195
		200
780	GTA CAC ATT CCC AAC AAC GAC AAG AAG GGA TTT TAT AAG AAA AAG CAG	220
	Val His Ile Pro Asn Cys Asp Lys Lys Gly Phe Tyr Lys Lys Lys Gln	
		210
		215
828	TGT CGC CCT TCC AAA GGC AGG AAG CGG GGC TTC TGC TGG TGT GTG GAT	240
	Cys Arg Pro Ser Lys Gly Arg Lys Arg Gly Phe Cys Trp Cys Val Asp	
		225
		230
		235

FIG. 13C

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876	AAG	TAT	GGG	CAG	CCT	CTG	CCA	GGC	TAC	ACC	ACC	AAG	GGG	AAG	GAG	GAC
	Lys	Tyr	Gly	Gln	Pro	Leu	Pro	Gly	Tyr	Thr	Thr	Lys	Gly	Lys	Glu	Asp
					245					250					255	
924	GTG	CAC	TGC	TAC	AGC	ATG	CAG	AGC	AAG	TAG						
	Val	His	Cys	Tyr	Ser	Met	Gln	Ser	Lys							
				260					265							

FIG. 13D



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10	20	30	40	50	60
123 456 789 012 345 678 901 234 567 890					
GGT GCT TCT TCT GCT GGT CTT GGA CCA GTT GTT CGT TGT GAA CCA TGT GAT GCA CGA GCT					60
TTA GCT CAA TGT GCT CCA CCA CCA GCT GTT TGT GCT GAA TTA GTT CGA GAA CCA GGT TGT					120
GGT TGT TGT TTA ACT TGT GCT TTA TCT GAA GGT CAA CCA TGT GGT ATT TAT ACT GAA CGT					180
TGC GGT AGT GGT TTG CGT TGT CAA CCA AGC CCA GAT GAA GCT AGG CCT TTA CAA GCA TTA					240
TTA GAT GGT CGA GGT CTG TGT GTT AAT GCG TCC GCT GTT TCT CGA TTG CGC GCT TAT TTA					300
TTA CCT GCC CCA CCG GCA CCG GGT AAC GCC TCC GAA AGC GAA GAG GAT CGT TCT GCG GGT					360
TCC GTT GAA TCT CCA AGT GTG AGT TCT ACC CAT CGA GTT AGC GAC CCG AAA TTT CAT CCG					420
TTG CAC TCT AAA ATC ATT ATT AAA AAG GGT CAC GCA AAG GAT TCT CAA CGT TAT AAG					480
GTG GAT TAT GAA AGC CAA TCT ACC GAC ACT CAA AAT TTT AGT AGT GAA AGT AAA CGT GAA					540
ACC GAG TAC GGC CCG TGT CGA CGT GAG ATG GAG GAT ACC TTA AAC CAT TTA AAA TTT TTG					600
AAC GTT TTA TCC CCG CGT GGC GTT CAT ATC CCG AAT TGC GAT AAA AAA GGC TTC TAC AAA					660
AAG AAA CAA TGC CGT CCG AGT AAG GGT CGT AAA CGA GGT TTT TGT TGG TGC GTT GAC AAA					720
TAC GGT CAA CCG TTG CCG GGT TAT ACT ACT AAA GGC AAA GAA GAT GTT CAT TGT TAT TCT					780
ATG CAA TCT AAA TAA TGC ATC TCG AGA ATT C					811

FIG. 14

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ATGCAGCGGGCGGACCCACGCTCTGGGCGGCTGGCTGACTCTGCTGGTGTCTGCTCCGCGGCGCGGCTGGCGCGGGCTGGC  
 MetGlnArgAlaArgProThrLeuTrpAlaAlaAlaLeuThrLeuLeuValLeuLeuArgGlyProProValAlaArgAlaGly  
 -1 +1

GCGAGCTCGGCGGGCTTGGGTCCCGTGGTGGCTGGAGCCGTGCGACGCGCGTGCACCTGGCCAGTGCAGCCGCTCCGCCGCGCTGTGGCGGGAGCTG  
 AlaSerSerAlaGlyLeuGlyProValValArgCysGluProCysAspAlaArgAlaLeuAlaGlnCysAlaProProAlaValCysAlaGluLeu

GTGCGGAGCGGGCTGCTGCTGACGTGGCAGTGCAGGAGGGCCAGCCGTGCGGCATCTACACGAGCGCTGTGGCTCCGGCCCTTCGCTGC  
 ValArgGluProGlyCysGlyCysLeuThrCysAlaLeuSerGluGlyGlnProCysGlyIleTyrThrGluArgCysGlySerGlyLeuArgCys

CAGCCGTGCGCCGACGAGCGCGACCGCTGCAGGCGCTGCTGGACGCGCGGGCTCTGCGTCAACGCTAGTGGCTCAGCCGCTGCGCGCCCTACCTG  
 GlnProSerProAspGluAlaArgProLeuGlnAlaLeuLeuAspGlyArgGlyLeuCysValAsnAlaSerAlaValSerArgLeuArgAlaTyrLeu

CTGCCAGCGCGCCAGCTCCAGGAAATGCTAGTGAGTCGGAGGAAGACCGCAGCGCGGCGAGTGTGGAGAGCCGTCGCTCCAGCAGCAGGGTG  
 LeuProAlaProProAlaProGlyAsnAlaSerGluSerGluGluAspArgSerAlaGlySerValGluSerProSerValSerSerThrHisArgVal

TCTGATCCCAAGTTCACCCCTCCATTCAAAGATAATCATCATCAAGAAAGGCCATGTCTAAAGACAGCCAGCGCTACAAAGTTGACTACGAGTCTCAG  
 SerAspProLysPheHisProLeuHisSerLysIleIleIleLysLysGlyHisAlaLysAspSerGlnArgTyrLysValAspTyrGluSerGln

FIG. 15A

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AGCACAGATACCCAGAACTTCTCCTCCGAGTCCAAGCGGGAGACAGAAATATGGTCCCTGCCGTAGAGAAATGGAAGACACACTGAATCACCTGAAGTTC  
SerThrAspThrGlnAsnPheSerSerGluSerLysArgGluThrGluTyrGlyProCysArgArgGluMetGluAspThrLeuAsnHisLeuLysPhe

CTCAATGTGCTGAGTCCCAGGGGTGTACACATTCCCAACTGTGACAAGAAGGATTTTATAAGAAAAGCAGTGTGCCCCCTCCAAAGGCAGGAAGCGG  
LeuAsnValLeuSerProArgGlyValHisIleProAsnCysAspLysLysGlyPheTyrLysLysLysGlnCysArgProSerLysGlyArgLysArg

GGCTTCTGCTGGTGTGGATAAGTATGGGCAGCCTCTCCAGGCTACACCACCAAGGGGAAGGACCGTGCACGTGCTACAGCATGCAGAGCAAGTAG  
GlyPheCysTrpCysValAspLysTyrGlyGlnProLeuProGlyTyrThrThrLysGlyLysGluAspValHisCysTyrSerMetGlnSerLys.

**FIG. 15B**

## INTERNATIONAL SEARCH REPORT

International application No

PCT/US95/08925

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) Please See Extra Sheet

US CL 514/12, 21

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. 514/12, 21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Spencer, "Modern Concepts of Insulin-like Growth Factors," published 1991 by Elsevier Science Publishing Co., Inc. (New York, NY), pages 381-383, see entire document.	1-22



Further documents are listed in the continuation of Box C.



See patent family annex.

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07 SEPTEMBER 1995

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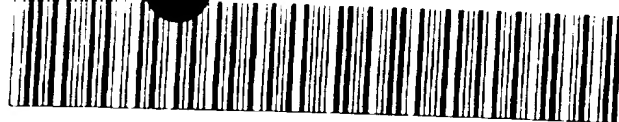
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International application No  
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A. CLASSIFICATION OF SUBJECT MATTER  
IPC (6)

C07K 14/00, 14/65; A61K 38/00, 38/28





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(54) Title: IGF/IGFBP COMPLEX FOR PROMOTING BONE FORMATION AND FOR REGULATING BONE REMODELING

(57) Abstract

IGF and IGFBP are administered to stimulate new bone formation in subjects with bone loss due to bone marrow disorders, connective tissue disorders, drugs, pregnancy, lactation, chronic hypophosphatemia, hyperphosphatasia, insulin-dependent diabetes mellitus, anorexia nervosa, cadmium poisoning, juvenile osteoporosis, Paget's disease of bone, osteoarthritis and periodontal disease. IGF-I and IGFBP-3 are optionally combined with agents that inhibit bone resorption.

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5           IGF/IGFBP COMPLEX FOR PROMOTING BONE FORMATION  
              AND FOR REGULATING BONE REMODELING

Technical Field

          The instant invention relates generally to  
10   polypeptide factors and their use in bone growth and  
     maturation. Specifically, the invention relates to the  
     use of a complex of insulin-like growth factor (IGF) and  
     insulin-like growth factor binding protein (IGFBP) with  
     or without an inhibitor of bone resorption to stimulate  
15   bone formation.

Background Art

          Growth factors are polypeptides which stimulate  
     a wide variety of biological responses (e.g., DNA  
20   synthesis, cell division, cell differentiation,  
     expression of specific genes, etc.) in a defined  
     population of target cells. A variety of growth factors  
     have been identified including transforming growth  
     factor- $\beta$ 1 (TGF- $\beta$ 1), TGF- $\beta$ 2, TGF- $\beta$ 3, epidermal growth  
25   factor (EGF), platelet-derived growth factor (PDGF),  
     fibroblast growth factor (FGF), insulin-like growth  
     factor-I (IGF-I), and IGF-II.

          IGF-I and IGF-II are related in amino acid  
     sequence and structure, with each polypeptide having a  
30   molecular weight of approximately 7500 daltons. IGF-I  
     mediates the major effects of growth hormone and thus is  
     the primary mediator of skeletal growth after birth.  
     IGF-I has also been implicated in the actions of various  
     other growth factors, since treatment of cells with such  
35   growth factors leads to increased production of IGF-I.

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Both IGF-I and IGF-II have insulin-like activity (hence the name) and are mitogenic (stimulating cell division) for various types of cells involved in the growth and differentiation of skeletal tissues such as muscle and bone, as well as non-skeletal tissues.

IGF can be measured in blood serum to diagnose abnormal growth-related conditions, e.g., gigantism, acromegaly, dwarfism, various growth hormone deficiencies, etc. Although IGF is produced in many tissues, most circulating IGF is believed to be synthesized in the liver.

Unlike most growth factors, the IGFs are present in substantial quantity in the circulation, but only a very small fraction of this IGF is free in the circulation or in other body fluids. Most IGF is complexed with IGF-binding proteins. IGF in the blood is mainly complexed with IGFBP-3, the major circulating IGF-binding protein. Almost all IGF circulates in a non-covalently associated ternary complex composed of IGF-I or -II, an IGF specific binding protein termed IGFBP-3, and a larger protein termed the Acid Labile Subunit (ALS). This ternary complex is composed of equimolar amounts of each of the three components. The ALS has no direct IGF binding activity and appears to bind only a pre-formed IGF/IGFBP-3 complex. The ternary complex of IGF + IGFBP-3 + ALS has a molecular weight of approximately 150,000 daltons. This ternary complex likely functions in the circulation "as a reservoir and a buffer for IGF-I and IGF-II preventing rapid changes of free IGF." Blum, W.F., et al., "Plasma IGFBP-3 Levels as Clinical Indicators", In Modern Concepts in Insulin-Like Growth Factors, E. M. Spencer, ed., Elsevier, New York, pages 381-393, 1991.

Having most circulating IGF in complexes is beneficial. Excess free IGF can cause serious

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hypoglycemia because IGF has insulin-like effects on circulating glucose levels. In contrast to the low levels of free IGFs and IGFBP-3, there is a substantial pool of free ALS in plasma which assures that IGF/IGFBP-3 complex entering the circulation immediately forms a ternary complex.

#### IGF Binding Proteins

IGFBP-3 is the most abundant IGF binding protein in the circulation. Recently, Wood et al. (Mol. Endocrin. (1988), 2:1176-85) and Spratt et al. (Growth Factors (1990), 3:63-72) described the cloning and expression of human IGFBP-3, whose structure is incorporated herein by reference. The gene for IGFBP-3 codes for 291 amino acids, the first 27 of which represent a characteristic signal sequence. Thus, the mature protein comprises 264 amino acids and has a predicted molecular weight of 28,749 (without glycosylation or other post-translational changes). When the human IGFBP-3 gene was expressed in Chinese hamster ovary ("CHO") cells and the conditioned culture medium was subjected to SDS electrophoresis and transferred to nitrocellulose membrane for ligand binding analysis, Spratt et al. reported "the presence of a 43-45 kd doublet, a 28 kd band and a minor 31 kd band" protein bands (p. 69), indicating there were post-translational changes. A side-by-side comparison revealed that the 43-45 kd doublet was also present in human serum.

Figures 12-15 show coding sequences and deduced amino acid sequences of IGFBP-3 suitable for various forms of use in the present invention.

It is unclear which tissue is the primary source of circulating IGFBP-3, although synthesis has been demonstrated in numerous cell types, including human fibroblasts, liver cells (most likely Kupfer cells) and

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osteoblasts. cDNA libraries that include the IGFBP-3 cDNA have been obtained from liver and other tissues. Vascular endothelial cells produce IGFBP-3 and may be the major source for systemic IGFBP-3.

5 IGFBP-3 has been purified from natural sources and produced by recombinant means. For instance, IGFBP-3 can be purified from natural sources using a process such as that shown in Martin and Baxter (J. Biol. Chem. (1986) 261:8754-60) IGFBP-3 also can be synthesized by  
10 recombinant organisms as discussed in Sommer, A. et al., In Modern Concepts of Insulin-Like Growth Factors, E. M. Spencer, ed., Elsevier, New York, pp. 715-728, 1991. This recombinant IGFBP-3 binds IGF-I with a 1:1 molar stoichiometry.

15 At least five other distinct IGF binding proteins have been identified in various tissues and body fluids. Although all these proteins bind IGFs, they each originate from separate genes and they have distinct amino acid sequences. Thus, the binding proteins are not  
20 merely analogs of a common precursor. For example, Spratt et al. compared the amino acid sequences of IGFBP-1, -2 and -3. Of the total 264 amino acids in the mature protein, only 28% of the amino acids are identical between IGFBP-3 and IGFBP-1, and 33% are identical  
25 between IGFBP-3 and IGFBP-2. Spratt et al. suggested that the similar portions of the binding proteins are the region(s) that bind IGF. Unlike IGFBP-3, the other IGFBPs in the circulation are not saturated with IGFs. All six known IGFBPs are reviewed and compared by  
30 Shimasaki and Ling, Prog. Growth Factor Res. (1991) 3:243-66.

Spencer et al. (1991) Bone 12:21-26; and Tobias et al. (1992) Endocrinology 131:2387-2392 report the stimulation of bone formation by IGF-I.

35

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The trabecular bone of rats, like that of humans, shows a coupling of bone formation to bone resorption, such that the increased resorption that occurs with estrogen deficiency entrains increased bone formation, which can be suppressed by inhibition of bone resorption. It has been established that in adult humans this coupling of formation and resorption involves a site specific sequence of events, in which bone resorption is normally followed, at the same site, by bone formation.

10 Frost (1985) Clin. Orthop. Rel. Res. 200:198-225.

There is also some evidence that bone formation can occur without previous bone resorption, primarily in those situations where demands for mechanical support of the skeleton are increased (modeling). Parfitt, A.M., et al. (1984) Calcif. Tissue Int. 36:5123-5128.

20 The present invention offers in vivo single or combination therapy for stimulating new bone formation through the administration of the IGF/IGFBP complex or through the administration of the IGF/IGFBP complex and an agent which inhibits bone resorption. These combinations provide more effective therapy for prevention of bone loss and replacement of bone.

#### Disclosure of the Invention

25 The present invention discloses the use of IGF and its binding protein for the initiation and promotion of bone formation and the regulation of bone remodeling. The IGF/IGFBP-3 complex can be used alone or in conjunction with an inhibitor of bone resorption.

30 The present invention discloses a method for stimulating bone formation in a subject who has a bone marrow disorder causing bone loss. The method comprises administering to the subject pharmaceutically effective doses of IGF-I and IGFBP-3, with or without an inhibitor  
35 of bone resorption.

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In another embodiment, the present invention discloses a method for stimulating bone formation in a subject who has a connective tissue disorder causing bone loss. The method comprises administering to the subject  
5 pharmaceutically effective doses of the IGF-I/IGFBP-3 complex.

In another embodiment, the present invention discloses a method for treating a subject who has drug-related osteoporosis. The method comprises administering  
10 to the subject pharmaceutically effective doses of the IGF-I/IGFBP-3 complex.

In another embodiment, the present invention discloses a method of stimulating bone formation in a subject who has bone loss due to pregnancy, lactation,  
15 chronic hypophosphatemia, hyperphosphatasia, insulin-dependent diabetes mellitus, anorexia nervosa, cadmium poisoning, juvenile osteoporosis. The method comprises administering to the subject pharmaceutically effective doses of the IGF-I/IGFBP-3 complex.

In another embodiment, the present invention discloses a method for stimulating bone formation in a subject who has periodontal bone loss. The method  
20 comprises administering to the subject pharmaceutically effective doses of IGF-I and IGFBP-3.

In another embodiment, the present invention discloses a method for stimulating bone formation in a subject who has bone loss associated with osteoarthritis,  
25 disuse, or prolonged exposure to reduced gravitational field. The method comprises administering to the subject pharmaceutically effective doses of IGF-I and IGFBP-3.  
30

In another embodiment, the present invention discloses a composition for inducing bone formation in a subject. The composition comprises IGF-I, IGFBP-3 and an  
35 inhibitor of bone resorption in a pharmaceutically acceptable excipient.

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Brief Description of the Figures

Figure 1 shows a schematic of the experimental design used in experiments carried out in support of the invention (Example 2);

5           Figures 2A and 2B show bar graphs of mean body weights (2A) and lean body mass (2B) of ovariectomized (OVX) animals treated for 8 weeks with rhIGF-I or rhIGF1/IGFBP-3 complex at the doses of IGF-I indicated, compared to no treatment (solid bars) and sham operated controls (solid line);

10

Figure 3 shows a bar graph of endochondral bone growth) of ovariectomized (OVX) animals treated for 8 weeks with rhIGF-I or rhIGF-I/IGFBP-3 complex at the doses of IGF-I indicated, compared to no treatment (solid bars) and sham operated controls (solid line);

15

Figures 4A and 4B show bar graphs of tibial periosteal bone formation rate (4A) and tibial endocortical resorption (4B) of ovariectomized (OVX) animals treated for 8 weeks with rhIGF-I or rhIGF-I/IGFBP-3 complex at the doses of IGF-I indicated, compared to no treatment (solid bars) and sham operated controls (solid line);

20

Figures 5A-5CC show matching UV micrographs (5A-5C, 3X magnification; 5CC, 12X magnification of periosteal envelope region shown in 5C) of ground 40  $\mu$ m thick tibial cross-sections from sham (5A), OVX (5B), and OVX rats treated with 7.5 mg/kg rhIGF-I complexed with rhIGFBP-3 (5C, 5CC) where new bone formation on the periosteal envelope is indicated by the arrows;

25

Figures 6A-6D show polarized light micrographs (12.5 X magnification) of unstained, 40  $\mu$ m thick cross sections from Sham operated animals (6A), OVX animals (6B), and from animals treated with 7.5 mg/kg rhIGF-I complexed with rhIGFBP-3 (6C, 6D), where outer lamellar

30

35

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layers are indicated by arrowheads and inner lamellar layers are indicated by arrows;

Figures 7A-7D show bar graphs of bone formation rates of cancellous bone in the metaphyses (7A),  
5 epiphyses (7B), lumbar vertebral bodies (7C) and femoral neck region (7D), of ovariectomized (OVX) animals treated for 8 weeks with rhIGF-I or rhIGF-I/IGFBP-3 complex with the doses of IGF-I indicated, compared to no treatment (solid bars) and sham operated controls (solid line),  
10 where an asterisk indicates  $p < 0.05$  compared to OVX group;

Figures 8A-8C show UV micrographs of cancellous trabeculae in distal femoral epiphysis from sham-operated control (8A), OVX (8B) and 7.5 ml/kg rhIGF-I/IGFBP-3  
15 complex (8C) treated rats, where arrows indicate resorption pits and arrowheads indicate newly formed bone;

Figures 9A-9C show matching micrographs (3X magnification) of 4  $\mu$ m section taken from distal femurs stained by the mineral-staining method of von Kossa from  
20 sham-operated controls (9A) OVX (9B) and 7.5 mg/kg rhIGF-I/IGFBP-3 complex treated rats (9C);

Figures 10A-10C show cross-sectional images (2.5X magnification) of sections taken from femoral mid-neck region of sham-operated control (10A), OVX rats  
25 (10B) and from rats treated with 7.5 mg/kg rhIGF-I complexed with IGFBP-3 (10C);

Figure 11 shows the amino acid sequences of human IGF-I used in the present invention (SEQ ID NO:1);

30 Figure 12 shows the amino acid sequence of human IGFBP-3 (SEQ ID NO:2), where an asterisk at position 5 indicates substitution of glycine for alanine according to naturally occurring heterogeneity in the sequence);

35



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Figure 13 shows a nucleotide sequence of human IGFBP-3 (SEQ ID NO:3 and SEQ ID NO:4) used to produce rhIGFBP-3 for use in accordance with the invention;

5 Figure 14 shows an alternate nucleotide sequence (SEQ ID NO:5) for production of rhIGFBP-3 for use in accordance with the invention; and

Figure 15 shows a second alternate nucleotide sequence (SEQ ID NO:6 and SEQ ID NO:7) for production of rhIGFBP-3 for use in accordance with the invention.

10

### Modes for Carrying Out the Invention

#### A. Definitions

It must be noted that as used in this specification and the appended claims, the singular forms  
15 "a", "an" and "the" include plural referents unless the context clearly dictates otherwise.

The term "connective tissue disorder" includes conditions such as osteogenesis imperfecta, Ehlers-Danlos syndrome, Marfans syndrome, cutis laxa, homocystinuria,  
20 Mankes's syndrome and scurvy. These have been reported to be associated with and cause bone loss.

"Drug-related osteoporosis" means osteoporosis whose only identified cause is a drug. The definition includes all known drugs which cause osteoporosis, as  
25 well as those drugs later discovered to cause osteoporosis. Examples of drugs known to cause osteoporosis include corticosteroids, heparin, oral anticoagulants, anticonvulsants, methotrexate, thyroid hormone, lithium and gonadotrophin-releasing analogs.

30 "Insulin-like growth factor (IGF)" comprises a family of factors, including but not limited to IGF-I and IGF-II. IGF is a polypeptide having a molecular weight of about 7500 daltons. IGF can be obtained from natural sources or prepared by recombinant means. Preferably IGF  
35 is IGF-I from human sources. Most preferably, IGF is

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is IGF-I from human sources. Most preferably, IGF is human IGF-I made by recombinant means, such as by the methods detailed in Example 3 herein, and designated rhIGF-I.

5 "Insulin-like growth factor binding protein (IGFBP)" comprises a family of binding proteins, including but not limited to IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 and IGFBP-6. Each IGFBP can be obtained from natural sources or prepared by recombinant means,  
10 such as detailed in Example 4 herein. At least one form of IGFBP (for example, IGFBP-3) complexes with IGF and with a third molecule known as ALS. IGFBP also can be a mixture of any combination of the six IGFBP's. Such a mixture would take advantage of the different binding  
15 affinities for IGF-I and/or IGF-II, the ability of some IGFBP's to bind to cell surfaces, and the different half-lives.

A "therapeutic composition" as used herein is defined as comprising the IGF binding protein (IGFBP)  
20 with IGF and a bone resorption inhibitor. The therapeutic composition can also contain excipients such as water, minerals and carriers such as protein.

The method of the present invention contemplates treating a subject in need of local bone  
25 repair or replacement with IGFBP in combination with IGF with or without a bone resorption inhibitor. The IGF may be any of the IGF family, including but not limited to, IGF-I and IGF-II, or a combination thereof. When IGF-I and IGF-II are combined, the ratio of IGF-I to IGF-II  
30 ranges from 0.01:1 to 99:1.

"IGFBP-1" is an IGF-binding protein whose molecular structure was disclosed by Brewer et al., Biochem. Biophys. Res. Comm. (1988) 152(3):1289-1297, and by Drop et al. in PCT Publication No. WO 89/98667,  
35 published on September 21, 1989, and is incorporated

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herein by reference. Human IGFBP-1 has 234 amino acids and a molecular weight of about 28 kd.

"IGFBP-2" comprises 289 amino acids (human) and has a molecular weight of 36 kd under nonreducing conditions. The amino acid sequence of human IGFBP-2 was determined from cDNA clones isolated from a human fetal liver library by Binkert et al. EMBO J. (1989) 8:2493-2502, and is incorporated herein by reference. IGFBP-2 also may bind to cell surfaces. IGFBP-2 has a preference for IGF-II, and thus is preferred in formulations comprising IGF-II.

"IGFBP-3" is the preferred IGFBP in the IGF/IGFBP complex. Native and recombinant IGFBP-3, as well as some N-terminal and C-terminal fragments, bind IGF-I and IGF-II. Human IGFBP-3 comprises 264 amino acids and has three potential N-linked glycosylation sites. IGFBP-3 is the major IGFBP in blood.

Nearly all IGF-I or IGF-II in blood is bound to IGFBP-3. The IGF/IGFBP-3 complex normally circulates in the form of a complex in humans and other mammals and avians. This complex associates with a third protein (ALS), which is present in excess over the concentration of IGF and IGFBP-3. Therefore, in the circulation, ALS is found both associated with the IGF/IGFBP-3 complex and in free form. The resultant ternary complex has a size of about 150 kD. Administration of a pre-formed complex of IGF and IGFBP-3, either from natural or recombinant sources, results in the formation of the ternary complex with the normally excess ALS. This type of treatment appears to produce a long term increase in the level of circulating IGF, which is gradually released from the ternary complex. This mode of administration avoids the detrimental side effects associated with administration of free IGF-I, e.g., hypoglycemia, suppression of growth hormone and ALS production, and release of endogenous

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IGF-II since administered exogenous free IGF-I replaces endogenous IGF-II in normally circulating IGF-II/IGFBP-3 complexes.

"IGFBP-4" and "IGFBP-6" are glycosylated proteins which are widely distributed in the body. The primary structure of IGFBP-4 was reported by Shimasaki et al. Mol. Endocrinol. (1990) 4:1451-1458, and is incorporated herein by reference. IGFBP-6, whose cDNA has been isolated by Shimasaki et al. (Mol. Endocrinol. (1991) 5:938-48), has a much greater affinity for IGF-II than for IGF-I and may be preferred with formulations containing IGF-II. This reference is incorporated herein by reference.

"IGFBP-5" is a 252-amino acid protein which is not glycosylated. Shimasaki et al. (J. Biol. Chem. (1991) 266:10646-53) cloned human IGFBP-5 cDNA from a human placenta library, and is incorporated herein by reference.

Depending on the binding, metabolic and pharmacokinetic characteristics required in the IGF/IGFBP complex formulation, these binding proteins can be added to the complex formulation in various proportions. These IGFBP's can be combined in a wide variety of ratios with IGF-I and/or IGF-II.

The term "inhibition of bone resorption" refers to prevention of bone loss, especially the inhibition of removal of existing bone either from the mineral phase and/or the organic matrix phase, through direct or indirect alteration of osteoclast formation or metabolism. Thus, the term "inhibitor of bone resorption" as used herein refers to agents that prevent bone loss by the direct or indirect alteration of osteoclast formation or metabolism.

The term "bone remodeling" refers to that process which renews the skeleton and repairs microdamage

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before it accumulates to the point of loss of skeletal integrity. Most adult human metabolic bone disease results from derangement of remodeling processes. The individual remodeling packets are known as "basic multicellular units" (BMU). Remodeling which occurs in BMUs follows a preprogrammed sequence: activation → resorption → formation (ARF). (Recker, R.R., "Bone Histomorphometry: Techniques and Interpretation." Boca Raton, FL, 1983, pp. 37-57.)

"Bone formation" includes increases in the number and/or activity of BMU (Basic Multicellular Units) producing new bone. This process can take place anywhere in the skeleton and affect cancellous and/or cortical bone with no regard to functional differences and turn-over rates which particular bone sites might have in the skeleton. (Frost (1986) Intermediary Organization of the Skeleton, Vols. I and II. CRC Press, Boca Raton, FL.)

The term "osteogenically effective" means that amount which affects the formation and differentiation of mature bone or early bone progenitor cells. As used herein, an osteogenically effective dose is also "pharmaceutically effective."

The term "subject" as used herein refers to a living vertebrate animal such as a mammal or bird in need of treatment, i.e., in need of bone repair or replacement. Such need arises in connective tissue disorders such as osteogenesis imperfecta, Ehlers-Danlos syndrome, Marfans syndrome, cutis laxa, homocystinuria, Menkes's syndrome and scurvy; osteoporosis related to such drugs as corticosteroids, heparin, oral anticoagulants, anticonvulsants, methotrexate, thyroid hormone, lithium and gonadotrophin-releasing analogs; pregnancy; lactation; chronic hypophosphatemia; hyperphosphatasia; insulin-dependent diabetes mellitus; anorexia nervosa; Paget's disease of bone; cadmium

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poisoning; periodontal disease; osteoarthritis, disuse of the musculoskeletal system such as occurs during prolonged bed rest, paralysis or semi-paralysis; and prolonged exposure to reduced gravitational field.

5           The term "treatment" as used herein shall mean (1) providing a subject with an amount of a substance sufficient to act prophylactically to prevent the development of bone loss; or (2) providing a subject with a sufficient amount of a substance so as to alleviate or  
10       eliminate the problems associated with the bone loss.

B.           General methods

Drugs which prevent bone loss and/or add back lost bone have been evaluated in the ovariectomized rat.  
15       This animal model is well established in the art (see, for example, Wronski et al. (1985) Calcif. Tissue Int. 37:324-328; Kimmel et al. (1990) Calcif. Tissue Int. 46:101-110; Durbridge et al. (1990) Calcif. Tissue Int. 47:383-387; and Miller et al. (1991) Bone 12:439-446;  
20       these references are hereby incorporated in their entirety). Wronski et al. ((1985) Calcif. Tissue Int. 43:179-183)) describe the association of bone loss and bone remodeling in the ovariectomized rat.

Examples of inhibitors of bone resorption  
25       include estrogen, such as conjugated estrogen, tamoxifen, bisphosphonates, calcitonins, or other small peptides or molecules that may inhibit bone resorption. (Turner et al. (1987) J. Bone Mineral Res. 2:115-122; Wronski et al. (1988) Endocrinology 128:681-686; and Wronski et al.  
30       (1989) Endocrinology 125:810-816; Pfeilshifter et al. (1987) Proc. Natl. Acad. Sci. U.S.A. 84:2024-2028; Turner et al. (1988) Endocrinology 122:1146-1150). An example of a small peptide is echistatin, which includes the arginine-glycine-aspartate (RGD) sequence which is  
35       recognized by some cell surface adhesion receptors and

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apparently disrupts osteoclast interaction (Fisher et al. (1993) Endocrinology 132: 1411-13). Another example of a bone resorption factor is OPF or osteoclastpoietic factor (PCT Publication WO 93/01827 published 4 February 1993).

5 Other agents that are under investigation or have been proposed to inhibit bone resorption include mithramycin, gallium nitrate, glucocorticoids, transforming growth factor- $\beta$  (TGF- $\beta$ ), interferon- $\gamma$  and amylin. (Zaidi et al. (October 1992) Curr. Opin. Therapeutic Patents, pp. 1517-  
10 38).

The entire molecule of a particular inhibitor may be used, or alternatively, only a functional part of the inhibitor molecule may be used. Bisphosphonates include, but are not limited to, pamidronate acid,  
15 alendronate, tiludronate, risedronate and other experimental compounds.

Certain growth factors can inhibit bone resorption, or are anti-resorptive. Examples of such growth factors include but are not limited to the  
20 transforming growth factors- $\beta$ .

In accordance with the method of the present invention, the formulation comprises a complex of IGF and IGFBP administered with or without a bone resorption inhibitor.

25 Preferably, the IGF is IGF-I, although IGF-II can be useful. Preferably, the IGFBP is IGFBP-3. Because IGF and IGFBP-3 naturally bind in a 1:1 molar ratio, a composition of equimolar amounts of IGF and IGFBP-3 is preferred. Nevertheless, the composition can  
30 be formulated with IGF:IGFBP-3 molar ratios ranging from 0.5:1 to 1.5:1. More preferably, the molar ratio is 0.9:1 to 1.3:1; and most preferably, the composition is formulated with approximately a 1:1 molar ratio.

In accordance with the method of the present  
35 invention, IGF and IGFBP-3 are human proteins obtained

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from natural or recombinant sources. Most preferably, IGF and IGFBP-3 are human IGF-I and IGFBP-3 made by recombinant means and designated rhIGF-I and rhIGFBP-3, respectively. rhIGFBP-3 can be in glycosylated or non-glycosylated form. *E. coli* is a source of the non-glycosylated IGFBP-3. Glycosylated IGFBP-3 can be obtained from CHO-cells.

The method of the present invention provides for formulating the complex in modes which are readily apparent to those skilled in the art. Preferably, the IGF and IGFBP-3 are complexed prior to administration to the treated individual. Preferably, the complex is formed by mixing approximately equimolar amounts of IGF-I and IGFBP-3 dissolved in physiologically compatible carriers such as normal saline solution or phosphate buffered saline solution. Most preferably, a concentrated solution of rhIGF-I and a concentrated solution of IGFBP-3 are mixed together for a sufficient time to form an equimolar complex.

Pharmaceutical compositions of the invention which include IGF, IGFBP and an inhibitor of bone resorption for administration include osteogenically effective amounts of IGF and IGFBP to promote bone formation and an inhibitory amount of a bone resorption inhibitor, in addition to a pharmaceutically acceptable excipient. Suitable excipients include most carriers approved for parenteral administration, including water, saline, Ringer's solution, Hank's solution, and solutions of glucose, lactose, dextrose, ethanol, glycerol, albumin, plasma, other protein-containing solutions and the like. These compositions may optionally include stabilizers, antioxidants, antimicrobials, preservatives, buffering agents, surfactants, and other accessory additives. The IGF/IGFBP complex and inhibitor of bone



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resorption may also be delivered in a slow release form from a suitable carrier.

Various vehicles may be used with the present invention. A thorough discussion of suitable vehicles for parenteral administration may be found in E.W. Martin, "Remington's Pharmaceutical Sciences" (Mack Pub. Co., current edition). Sections relating to the excipient vehicles and formulating are incorporated herein by reference. Such formulations are generally known to those skilled in the art and are administered systemically to provide systemic treatment.

The IGF/IGFBP complex and an agent which inhibits bone resorption may be administered simultaneously, as a single composition to the subject, or sequentially. If administered sequentially, the period between the administration of the IGF/IGFBP complex and the inhibitor of bone resorption is typically one day to one year, and preferably, one week to six months. If the IGF/IGFBP complex and the agent which inhibits bone resorption are administered as a single composition, the molar ratio of IGF/IGFBP complex to inhibitor of bone resorption varies considerably depending on the type of bone resorption inhibitor and the formulation of the IGF/IGFBP complex. The ratio for most compounds is between about 100:1 to 1:100. Furthermore, if administered as a single composition, the IGF/IGFBP complex and the inhibitor of bone resorption may be administered as separate molecules in the composition, or the respective molecules may be conjugated or fused according to techniques well known in the art.

The precise dosage necessarily varies with the age, size, sex and condition of the subject, the nature and severity of the disorder to be treated, and the like. Thus, a precise effective amount cannot be specified in

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advance and must be determined by the care giver. However, appropriate amounts have been and can be determined by routine experimentation with animal models, as described below.

5           In general terms, an effective dose of IGF/IGFBP complex for systemic treatment will range from about 1  $\mu$ g to about 10 mg IGF/kg of body weight. An effective dose for an inhibitor of bone resorption depends upon the particular inhibitor selected for  
10 administration. For example, an effective dose of estrogen is about 0.25 to 1.5 mg/day. An effective dose for bisphosphonates varies but is generally between about 0.05  $\mu$ g/kg to about 15 mg/kg of body weight. An effective dose for calcitonin is about 0.05 IU  
15 (International Units or Medical Research Council Units)/kg to about 2.5 IU/kg of body weight.

Effective doses for local administration range from about 0.01  $\mu$ g to 1 mg of IGF/IGFBP complex.

The methods and compositions of the invention  
20 are useful for treating bone fractures, defects, and disorders which result in weakened bones. Among these disorders are bone marrow dyscrasias such as plasma cell dyscrasias, leukemia, lymphomas, systemic mastocytosis, anemias, lipidoses and mucopolysaccharidoses; connective  
25 tissue disorders such as osteogenesis imperfecta, Ehlers-Danlos syndrome, Marfans syndrome, cutis laxa, homocystinuria, Menkes's syndrome and scurvy; osteoporosis related to such drugs as corticosteroids, heparin, oral anticoagulants, anticonvulsants,  
30 methotrexate, thyroid hormone, lithium and gonadotrophin-releasing analogs; pregnancy; lactation; chronic hypophosphatemia; hyperphosphatasia; insulin-dependent diabetes mellitus; anorexia nervosa; Paget's disease of bone; juvenile osteoporosis; cadmium poisoning;  
35 periodontal disease; and osteoarthritis. In addition,

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methods and compositions of the present invention are useful in treating weakened bones resulting from disuse of the musculoskeletal system, such as occurs as a consequence of prolonged bed rest or exposure to  
5 decreased gravitational fields.

In accordance with one method of use, the IGF/IGFBP complex may be administered locally to a specific area in need of bone growth or repair, with either the concomitant administration of the inhibitor of  
10 bone resorption at the site, or the administration of the inhibitor of bone resorption in a separate vehicle. Thus, the IGF/IGFBP complex and/or inhibitor of bone resorption may be implanted directly at the site to be treated, for example, by injection or surgical  
15 implantation in a sustained-release carrier. Suitable carriers include hydrogels, controlled- or sustained-release devices (e.g., an Alzet® minipump), polylactic acid, and collagen matrices. Presently preferred carriers are formulations of atelopeptide collagen  
20 containing particulate calcium phosphate mineral components, such as combinations of homologous or xenographic fibrillar atelopeptide collagen (for example Zyderm® Collagen Implant, available from Collagen Corporation, Palo Alto, California) with hydroxyapatite-  
25 tricalcium phosphate (HA-TCP, available from Zimmer, Inc., Warsaw, IN). It is presently preferred to administer implant compositions containing an IGF/IGFBP complex and/or an inhibitor of bone resorption in a collagen/mineral mixture implant.

30 IGF/IGFBP complex and/or an inhibitor of bone resorption, delivered in sustained-release vehicles is also particularly useful for improving implant fixation, for example, for improving ingrowth of new bone into a metal prosthesis in joint reconstruction and dental or  
35 orthopedic implants. Alternatively, the IGF/IGFBP

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complex may be delivered in the implant, with the inhibitor delivered in a separate vehicle, and vice versa.

Dental and orthopedic implants can be coated with the IGF/IGFBP complex in combination with an inhibitor of bone resorption to enhance attachment of the implant device to the bone. Alternatively, the IGF/IGFBP complex can be used to coat the implant, and the inhibitor of bone resorption can be administered concomitantly or sequentially in a separate vehicle, and vice versa.

In general, implant devices may be coated with the IGF/IGFBP complex and/or an inhibitor of bone resorption as follows. The IGF/IGFBP complex (and the inhibitor of bone resorption, if desired) is dissolved at a concentration in the range of 0.01 mg/ml to 200 mg/ml in phosphate-buffered saline (PBS) containing 2 mg/ml serum albumin. The porous end of an implant is dipped in the solution and is air dried (or lyophilized) or implanted immediately into the bony site. The viscosity of the coating solution is increased, if desired, by adding hyaluronate at a final concentration of 0.1 mg/ml to 100 mg/ml or by adding other pharmaceutically acceptable excipients. Alternatively, the solution containing the IGF/IGFBP complex (and the inhibitor of bone resorption, if desired) is mixed with collagen gel or human collagen (e.g. Zyderm® Collagen Implant, Collagen Corp., Palo Alto, CA) to a final collagen concentration of 2 mg/ml to 100 mg/ml to form a paste or gel, which is then used to coat the porous end of the implant device. The coated implant device is placed into the bony site immediately or is air dried and rehydrated with PBS prior to implanting, with the objective of maximizing new bone formation into the implant while

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minimizing the ingrowth of soft tissue into the implant site.

C. Examples

5           The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to extract, isolate, formulate and use the compositions and methods of the invention and are not intended to limit the scope  
10 of what the inventors regard as their invention. Efforts have been made to insure accuracy with respect to numbers used (e.g., amounts, times, temperature, etc.), but some experimental error and deviations should be accounted for. Unless indicated otherwise, parts are parts by  
15 weight, temperature is in degrees centigrade, pressure is at or near atmospheric, and other parameters are conventional and in accordance with those normally accepted by those skilled in the art.

20                           Example 1

          This example shows the use of free IGF-I and the IGF-I/IGFBP-3 complex in female rats with ovariectomy-induced osteoporosis. In this experiment, rats were treated with human recombinant IGF-I and  
25 IGFBP-3. The rhIGF-I (Ciba-Geigy) was synthesized in yeast and provided in sterile water and stored at -70°C. The rhIGFBP-3 was made according to the procedure described in Example 4 (Celtrix Pharmaceuticals, Inc., Santa Clara, CA) and was dissolved in phosphate-buffered  
30 saline and stored at -70°C until use. Prior to administration, these solutions were thawed, and sufficient amounts of IGF-I and IGFBP-3 were mixed to provide equimolar amounts of the two proteins. This experiment demonstrates the ability of the IGF-I/IGFBP-3

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complex to increase trabecular bone mass and other parameters in ovariectomized rats.

In this example, young female rats of 90-100 g body weight were ovariectomized by the dorsal route and were divided into six groups of eight rats each. An additional group consisted of eight intact, age-matched sham operated control rats. Six weeks after ovariectomy, rats were treated with vehicle or with one of the following combinations of IGF-I and IGFBP-3 or IGF alone, as indicated. In these experiments, the amounts of IGF and IGFBP used were calculated to provide a 1:1 molar ratio of IGF:IGFBP:

- Group 1: Sham Operated Controls; Vehicle
- Group 2: Ovariectomized Controls; Vehicle
- Group 3: Ovariectomized; 2.5 mg/kg IGF-I complexed to IGFBP-3 (9.5 mg/kg)
- Group 4: Ovariectomized; 0.25 mg/kg IGF-I complexed to IGFBP-3 (0.95 mg/kg)
- Group 5: Ovariectomized; 0.025 mg/kg IGF-I complexed to 0.095 mg/kg IGFBP-3
- Group 6: Ovariectomized; 2.5 mg/kg IGF-I
- Group 7: Ovariectomized; 0.25 mg/kg IGF-I

The complex was formed by mixing equimolar amounts of IGFBP-3 (dissolved in phosphate buffered saline (PBS), pH 6.0) and IGF-I (dissolved in 10 mM sodium acetate, pH 5.5) in the minimum volume feasible, and incubating the mixture overnight at 4°C. The complex was then diluted with PBS, pH 6.0, containing 0.1% rat serum albumin. The solutions were divided into aliquots containing the amount of material needed for one day, and stored at -70°C until needed. The controls received the dilution buffer.

The rats were treated for 22 days. The test substances were administered six times per week by one

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daily subcutaneous injection. One day before treatment was started and on the 17th day of treatment, 20 mg/kg of calcein was given by intraperitoneal injection. Calcein and tetracycline are markers for bone mineralization and are used to estimate the amount of bone formation between administrations. Similarly, on the tenth day 20 mg/kg of demeclocycline was administered. On day 23, 24 hours after the last injection, the rats were killed by anesthesia with carbon dioxide.

Body weights were recorded throughout the experiment. At autopsy, 0.1 ml of blood was taken for the determination of blood glucose. Serum was prepared from the rest of the blood, and total serum IGF-I levels were determined by radioimmunoassay (RIA). Gastrocnemius muscle, periuterine fat and uterus were removed, dissected free of connective tissue, and weighed.

The amount of trabecular and cortical bone was determined according to Gunness-Hey ((1984) Metab. Bone Dis. & Rel. Res. 5:177-81), incorporated herein by reference. Briefly, the femurs were cut in half at the mid-diaphysis using a dental saw. The proximal halves were discarded. The epiphysis of the distal half was cut off using a scalpel, and the bone was split into sagittal halves. The marrow was flushed out with water. With a dental curette, the metaphyseal trabecular bone was scraped out of both cortical shells, combined and put into 5% trichloroacetic acid (TCA). The two pieces of the remaining cortical bone were also combined and put into a separate tube with 5% TCA. After this preparation stood 16 hours at room temperature, the TCA extract was used for the determination of calcium by atomic absorption spectroscopy. The remaining demineralized matrix was washed successively with ethanol and methylene chloride, and dried under vacuum. After determination of the dry weight, the matrix was hydrolyzed with 6 M HCl at

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120°C for 5 hours. In the hydrolysate hydroxyproline was determined by a standard colorimetric assay (Jamall et al. (1981) Analyt. Biochem. 112:70-75).

5 The results of this experiment are detailed in Tables 1 and 2 and summarized below.

When ovariectomized ("Ovx" in the tables) control rats were compared to sham operated control animals, no significant differences were observed in cortical bone weight or hydroxyproline (Table 2).  
10 However, cortical bone calcium was significantly increased in ovariectomized rats (7%). This increase may be due to increased longitudinal bone growth and increase in organ size. In the ovariectomized rats, trabecular bone weight, calcium and hydroxyproline were reduced by  
15 65% (Table 1).

In ovariectomized rats treated with IGF-I alone, both doses (2.5 mg/kg and 0.25 mg/kg) increased trabecular bone mass; however, the lower dose was more effective than the higher dose. The lower dose increased  
20 various parameters 79%-118%; whereas, the higher dose increased various parameters by 57%-67%. There were marginal changes in other parameters.

Ovariectomized rats treated with IGF-I/IGFBP-3 complex showed an increase in various trabecular bone  
25 parameters (Table 1). Significant increases in trabecular calcium, dry weight, and hydroxyproline were observed at an IGF-I/IGFBP-3 dose of 12 mg/kg (2.5 mg/kg IGF-I and 9.5 mg/kg IGFBP-3). Cortical bone matrix dry weight was also increased significantly by treatment with  
30 12 mg/kg IGF-I/IGFBP-3 complex (Table 2).



Table 1Treatment Groups

	Group 1 (Sham Operated)	Group 2 (Ovx Control)	Group 3	Group 4	Group 5	Group 6	Group 7
IGF-I (mg/kg)	-	-	2.5	0.25	0.025	2.5	0.25
IGFBP-3 (mg/kg)	-	-	9.5	0.95	0.095	-	-

Trabecular Bone<sup>1</sup>

Calcium (mg) (%)	4.0±0.4 (+186)*	1.4±0.1 -	2.7±0.2 (+93)	1.8±0.2 (+29)	1.6±0.1 (+14)	2.2±0.2 (+57)*	2.5±0.2 (+79)*
Dry Weight (mg) (%)	2.9±0.4 (+164)*	1.1±0.1 -	2.1±0.2 (+91)	1.7±0.2 (+55)	1.6±0.3 (+45)	1.8±0.1 (+64)*	2.4±0.2 (+118)*
Hydroxyproline (mg) (%)	269±35 (+186)*	94±11 -	221±20 (+135)*	122±20 (+30)	157±30 (+67)	157±30 (+67)	169±16 (+80)*

<sup>1</sup> % are compared to ovariectomized, vehicle treated control values (Group 2)  
 \* p < 0.05 compared to Ovx control

Table 2Treatment Groups

	Group 1 (Sham Operated)	Group 2 (Ovx Control)	Group 3	Group 4	Group 5	Group 6	Group 7
IGF-I (mg/kg)	-	-	2.5	0.25	0.025	2.5	0.25
IGFBP-3 (mg/kg)	-	-	9.5	0.95	0.095	-	-

Cortical Bone<sup>1</sup>

Calcium (mg) (%)	24.6±0.5 (-7)*	26.5±0.7	26.1±0.9 (-2)	26.7±0.7 (+1)	23.2±0.5 (-12)*	24.0±0.6 (-9)*	23.5±0.6 (-11)*
Dry Weight (mg) (%)	52.8±3.4 (-7)	56.8±2.7	64.7±2.7 (+14)*	56.8±3.2 (±0)	59.1±2.0 (+4)	6.81±3.6 (+20)*	55.1±0.2 (-3)
Hydroxyproline (mg) (%)	2679±177 (-8)	2925±143	3144±146 (+7)	2978±157 (+2)	3050±107 (+4)	3331±102 (+14)*	3141±194 (+7)

<sup>1</sup> % are compared to ovariectomized, vehicle treated control values (Group 2)  
 \* p < 0.05 compared to Ovx control

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Example 2

Thirteen week old female Sprague Dawley rats (Charles River Associates) were housed for approximately three weeks before undergoing either ovariectomy (OVX) or sham operation (Sham). All OVXs were successful as determined by uterine weights measured at sacrifice. Eight weeks after surgery (designated day 0) the rats underwent a Dual Energy X-ray Absorptometry (DEXA) spine scan for baseline measurements of bone mineral density of vertebrae L3 through L6 (BMD) and were assigned into groups (see below). Attempts were made to exclude the "outliers" and have similar mean spine BMD values and body weights among the various groups.

The rhIGF-I/IGFBP-3 was prepared by mixing equimolar quantities of rhIGF-I and rhIGFBP-3. The vehicle employed for both rhIGF-I and rhIGF-I/IGFBP-3 was phosphate buffered saline (PBS), pH. 6.0.

Treatments (by daily subcutaneous injection) began the day after the baseline DEXA spine measurements (day 1) and continued for 56 days. Two additional "pretreatment" groups of rats (OVX and Sham) were sacrificed on day 0 to provide baseline data.

The rats were divided into seven groups according to the experimental paradigm illustrated in Fig. 1:

- (1) Sham rats treated with saline ("Sham control"; n=9).
- (2) OVX rats treated with saline ("OVX control"; n=11).
- (3) OVX rats treated with 0.9 mg/kg/day of rhIGF-I (n=7).
- (4) OVX rats treated with 2.6 mg/kg/day of rhIGF-I (n=7).
- (5) OVX rats treated with 0.9 mg/kg/day of rhIGF-I in equimolar ratio with IGFBP-3 (n=8).

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(6) OVX rats treated with 2.6 mg/kg/day of rhIGF-I in equimolar ratio with IGFBP-3 (n=8).

(7) OVX rats treated with 7.5 mg/kg/day of rhIGF-I in equimolar ratio with IGFBP-3 (n=8).

5 a. Serum Measurements

On day 22 food was withdrawn from the rats at about 7 a.m. and 150  $\mu$ l of blood was obtained from the tail of each rat under isoflurane anesthesia between 10 a.m. and noon. Immediately after the blood was  
10 collected, each rat received its normal daily treatment injection, and a second blood sample was obtained exactly two hours after the injection. Food was returned to the rats only after this second bleeding. Plasma glucose levels were measured by a standard colorimetric assay  
15 involving the oxidation of O-dianisidine by the peroxide produced from glucose as a result of treatment with glucose oxidase according to standard procedures well known in the art. (Table 3)

During the seventh week of the study, blood was  
20 again obtained from the tail during the normal DEXA scan. These samples were analyzed for glycosylated hemoglobin levels using an affinity chromatographic procedure, specifically the "GLYCO-TEK" assay kit (Helena Laboratories, Beaumont, TX) with the eluted hemoglobins  
25 detected by their absorbances at 405 nm.

Serum obtained at sacrifice was analyzed for levels of total IGF-I, rhIGF-I and rhIGFBP-3. Serum IGF-I levels were determined by two separate assays. Each assay was performed once on the entire set of  
30 samples.

First, a Nichols Institute RIA procedure was employed to quantitate the combined concentrations of endogenous rat IGF-I and the injected rhIGF-I. Samples of 80  $\mu$ l were extracted with 900  $\mu$ l of 87.5% 2N HCl/12.5%  
35 ethanol, centrifuged, and then 200  $\mu$ l of the supernatant

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was neutralized with 100  $\mu$ l of 855 mM Tris buffer pH 11.0. This neutralized extract was kept at -20°C for one hour, centrifuged, and then diluted 31-fold with phosphate buffered saline (PBS) prior to analyzing 50  $\mu$ l by RIA. A preliminary assay indicated that serum obtained from hypophysectomized rats (n=6) gave values below the limit of detection (66 ng/ml) and the assay gave a linear response when the volume of serum was varied from 10 to 100  $\mu$ l.

The second assay quantitated the serum levels of rhIGF-I by immunoradiometric assay (IRMA), according to protocols provided by the manufacturer, with a sample size of 10  $\mu$ l. A pooled rat serum sample containing 555 ng/ml IGF-I by the Nichols Institute RIA procedure gave values below the limit of detection (50 ng/ml) of this assay. In addition, serum levels of rhIGFBP-3 on 10  $\mu$ l samples were determined by RIA. The assay kits and procedures for both rhIGF-I and rhIGFBP-3 were obtained from Diagnostic Systems Laboratories (Webster, TX).

Serum IGF-I and IGFBP-3 levels and blood glycosylated hemoglobin values are shown in Table 4.

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**Table 3**

Fasting Plasma Glucose (mg/dl)  
(Day 22 of Study)

Group	Treatment	Time = 0 hours		Time = 2 hours		Student's P Value
		Glucose	SEM	Glucose	SEM	
1	Sham	191	6	162	6	0.003
2	OVX-Saline	183	8	197	4	0.120
3	0.9 mg/kg IGF-I	179	8	146	24	0.223
4	2.6 mg/kg IGF-I	163	6	73	11	0.001
5	0.9 mg/kg IGF-I + IGFBP-3	175	7	195	9	0.093
6	2.6 mg/kg IGF-I + IGFBP-3	192	6	204	10	0.349
7	7.5 mg/kg IGF-I + IGFBP-3	174	6	161	14	0.432

Table 4

Serum IGF-I and IGFBP-3 Levels  
and Blood Glycosylated Hemoglobin Values

Group	Treatment	Total IGF-I (ng/ml)	rhIGF-I (ng/ml)	rhIGFBP-3 (ng/ml)	Glycosylated Hemoglobin (%)
1	Sham-operated + Saline	not measured	not measured	not measured	6.7±0.3 (9)
2	OVX-Saline	487±33 (9)	<50 (8)	<250 (8)	7.2±0.2 (11)
3	0.9 mg/kg rhIGF-I	619±73 (5)	204±56 (5)	<250 (5)	7.3±0.3 (7)
4	2.6 mg/kg rhIGF-I	633±75 (7)	329±61 (7)	<250 (7)	7.3±0.3 (7)
5	0.9 mg/kg rhIGF-I/ IGFBP-3	1026±162 (6)	1141±294 (6)	2441±775 (6)	7.3±0.4 (8)
6	2.6 mg/kg rhIGF-I/ IGFBP-3	988±63 (7)	1391±10 (7)	2372±525 (7)	7.3±0.4 (8)
7	7.5 mg/kg rhIGF-I/ IGFBP-3	1783±273 (8)	2103±123 (8)	8217±2247 (8)	7.3±0.4 (8)
ANOVA P Value (Dose)		<0.001	-	-	0.83
ANOVA P Value (Treatment)		0.014	-	-	0.69

Data are presented as means ± SEM for the number of values indicated in parentheses. A two-factor ANOVA was performed to determine the effects of dose (rhIGF-I and rhIGF-I/IGFBP-3 groups) and treatment (rhIGF-I versus rhIGF-I/IGFBP-3 groups.)

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TABLE 4A  
(page 1 of 2)

Group	Treatment	Day 0		Day 14		Day 28	
		BMD	SEM	BMD	SEM	BMD	SEM
A	Pretreatment Sham	237	4	ND		ND	
B	Pretreatment OVX	215	4	ND		ND	
1	Sham - Saline	239	5	242	6	243	3
2	OVX - Saline	216	2	212	3	212	3
3	0.9 mg/kg IGF-I	211	4	204	5	201	2
4	2.6 mg/kg IGF-I	214	3	201	4	206	3
5	0.9 mg/kg IGF-I + IGFBP-3	213	4	210	5	211	4
6	2.6 mg/kg IGF-I + IGFBP-3	215	5	209	5	204	6
7	7.5 mg/kg IGF-I + IGFBP-3	219	5	215	4	214	5



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TABLE 4A  
(Page 2 of 2)

	<u>Day 42</u>		<u>Day 56</u>		<u>Change</u> <u>Days 0-56</u>	
	<u>BMD</u>	<u>SEM</u>	<u>BMD</u>	<u>SEM</u>	<u>BMD</u>	<u>SEM</u>
	ND		ND		ND	
	ND		ND		ND	
243	6		241	5	3	5
213	3		208	4	-8	3
199	4		199	5	-12	2
202	5		201	5	-12	7
212	4		211	4	-2	4
206	5		210	4	-5	3
213	4		219	4	0	3

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b. Bone Measurements

Bone surfaces undergoing active mineralization were labeled in all animals included in this study with the fluorescent bone marker declomycin and calcein by  
5 injecting those markers on days 9 and 2 prior to sacrifice, respectively.

The rats were sacrificed by exsanguination under ketamine/xylazine anesthesia. Numerous soft  
10 tissues were collected for histological analyses and some of these tissues were also weighed to examine organ hypertrophy. The long bones (tibia and femur), the spine and jaws were fixed or frozen for DEXA, mechanical and bone histomorphometric analyses according to the  
15 procedures detailed below.

1. DEXA Measurements.

Spine DEXA scans were performed on days 0 (described above), 14, 28, 42, and 56 (the day before  
20 sacrifice). DEXA whole-body scans (excluding the head and tail) for measurements of body composition (lean body mass and fat mass) were performed on days 7, 21, 35, and 49.

The DEXA measurements were made with a Hologic QDR-1000/W instrument according to directions provided by  
25 the manufacturer. The rats were anesthetized with a gaseous anesthetic of 2% isoflurane in oxygen during the scans and body weights were also obtained while the rats were anesthetized.

Parameters measured on the tibia and femurs  
30 during the DEXA analyses included length (mm), projected area ("global area") ( $\text{cm}^2$ ), global bone mineral content (BMC) (mg), global bone marrow density (BMD) ( $\text{mg}/\text{cm}^2$ ), global bone mineral apparent density (global BMAD = BMC divided by projected area to the 1.5 power) ( $\text{mg}/\text{cm}^3$ ),  
35 metaphyseal BMD (met BMD) ( $\text{mg}/\text{cm}^2$ ), cortical BMD

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(mg/cm<sup>2</sup>), and epiphyseal BMD (epiph BMD) (mg/cm<sup>2</sup>). The results are shown in Table 5 (tibia) and Table 6 (femur). In these tables, the numerical group designations correspond to those shown in Fig. 1. Groups A and B were taken for baseline measurements prior to treatment.

DEXA bone measurements were validated by comparing mineral content of the tibia as measured by both global tibia BMC values and ash weight. DEXA body composition measurements were validated by comparing the changes in body weight measured by both weighing on a balance and DEXA determinations of total body mass.

Table 5

DEXA Parameters on Excised Tibia																		
Group	Treatment	Length		Global Area		Global BMC		Global BMD		Global BMAD		Met BMD		Cortical BMD		Epiph BMD		
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
A	Sham - Pretreatment	39.8	0.4	1.41	0.02	323	7	229	3	193	2	241	3	216	3	287	6	
B	OVX - Pretreatment	40.4	0.1	1.39	0.02	286	6	206	2	175	2	187	4	211	3	235	2	
1	Sham - Saline	40.3	0.4	1.44	0.04	310	9	221	2	184	2	217	6	209	3	276	5	
2	OVX - Saline	40.7	0.6	1.46	0.02	313	7	215	3	178	2	198	3	221	3	231	3	
3	0.9 mg/kg IGF-I	40.4	0.4	1.39	0.04	284	12	205	4	174	2	192	5	210	6	217	6	
4	2.6 mg/kg IGF-I	41.7	0.4	1.48	0.02	316	7	214	3	176	3	196	5	221	4	226	4	
5	0.9 mg/kg IGF-I + IGFBP-3	41.2	0.3	1.46	0.03	306	7	210	2	174	3	197	4	215	3	223	5	
6	2.6 mg/kg IGF-I + IGFBP-3	40.8	0.5	1.50	0.04	332	10	223	3	182	3	200	4	227	2	237	4	
7	7.5 mg/kg IGF-I + IGFBP-3	42.0	0.2	1.59	0.02	355	4	223	3	177	3	204	3	233	3	238	4	

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Table 6

## DEXA Parameters on Excised Femurs

Group	Treatment	Length		Global Area		Global BMC		Global BMD		Global BMAD		Met BMD	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
A	Sham - Pretreatment	36.1	0.3	1.68	0.02	419	10	250	3	193	2	226	4
B	OVX - Pretreatment	35.9	0.3	1.60	0.02	360	7	223	3	175	3	172	5
1	Sham - Saline	36.5	0.3	1.71	0.03	429	15	250	5	191	2	212	5
2	OVX - Saline	36.9	0.3	1.75	0.03	401	10	229	2	173	1	177	3
3	0.9 mg/kg IGF-I	36.2	0.4	1.69	0.04	370	15	218	4	168	2	167	4
4	2.6 mg/kg IGF-I	37.6	0.4	1.78	0.02	411	10	231	3	173	1	173	3
5	0.9 mg/kg IGF-I + IGFBP-3	37.0	0.3	1.76	0.03	394	10	224	4	169	3	169	5
6	2.6 mg/kg IGF-I + IGFBP-3	37.4	0.3	1.80	0.04	424	13	235	4	175	2	174	4
7	7.5 mg/kg IGF-I + IGFBP-3	37.9	0.2	1.86	0.02	451	7	243	2	178	2	174	3

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## 2. Bone Histomorphometry and Structure

Right femurs, tibias and lumbar vertebral bodies (Lvb; L<sub>4</sub>-L<sub>5</sub>) were collected at necropsy, cleaned of soft tissues, fixed in 70% ethanol for 48h, dehydrated through upgraded ethanols, and embedded undecalcified in methyl methacrylate. Longitudinal sections of distal femoral epiphyses, metaphyses, Lvb, and cross-sections of the mid femoral neck and the distal tibial diaphyses proximal to the tibio-fibular junction were cut with a Reichert-Jung supercut microtome (Reichert-Jung, Heidelberg, Germany) to 15  $\mu$ m in thickness. Three consecutive sections were left unstained for dynamic histomorphometric analyses and the other three sections were stained by the von Kossa method for mineral, counterstained with toluidine blue, and analyzed for static histomorphometric parameters. The 100  $\mu$ m thick cross-sections from femoral midshaft and mid-neck region were cut with a precision low-speed bone saw (Isomet, Buehler, Lake Bluff, IL), glued to plastic slides, ground and polished to ~40  $\mu$ m in thickness (Ecomet 3, Buehler, Lake Bluff, IL) and analyzed for bone dynamic parameters. The same set of slides were used for static bone histomorphometry after being stained with the von Kossa method and covered with cover slips. Bone dynamic measurements were performed under UV light and 20-fold magnification by using a semi-automated software program called "Stereology" (KSS Computer Engineers, Magna, UT) described earlier by Miller et al. (1989) Bone 7:283-287. Static bone analyses were performed with an automated television microscope image analysis system and analyzed using "Image Analysis" software (KSS Computer Engineers, Magna, UT).

For the femoral neck only, internal structure of trabecular bone, interconnectivity, and connectivity between endocortical surface and trabecular network, the

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"Nodal" and "Star volume" analyses were performed as described by Miller and Wronski (1993) Anat. Record 236:433-441 and Bagi and Miller (1994) Anat. Record 239:1-12. The marrow star volume is a direct measure of trabecular separation, while the nodal analysis describes the number of nodes and struts, type of struts, and number of inter-trabecular connectivity. Connections between endocortical surface and trabecular network are important determinants of bone strength linking cortical and cancellous bone into one anatomico-functional unit. The total number of endocortico-trabecular connections was counted and the percentages of free ending trabeculae versus those connected with other trabeculae and/or the endocortical surface were calculated. All measurements and derived parameters describing bone dynamic and structure were performed as recommended by ASBMR Histomorphometric Committee (Parfitt et al. (1987) J. Bone Min. Res. 2:595-610.

### 3. Bone Failure Strength and Internal Cortical Bone Structure

Left femurs were cleaned of soft tissue and were used in DEXA measurements of bone mineral density (BMD) and bone mineral content (BMC). To test the biomechanical properties of the cortical bone, the proximal and distal ends of each femur were secured in test fixtures, designed to allow freedom of motion parallel to the long axis of the bone. The fixtures were mounted in an MTS 858 Bionix System (MTS System Corp., Minneapolis, MN) and torque was measured with an externally conditioned miniature torque transducer (range 0-50 in-lbs; Model QWFK-8M/1941, Sensotec, Columbus, OH) as described earlier (van der Meulen, M.C.H., "Bone Strength in Young, Suspended Rats, Stanford University Thesis, 1993). (Mechanical tests were performed at the

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Department of Mechanical Engineering, Stanford University, Stanford, CA; and Rehabilitation Research and Development Center, Palo Alto, CA; and VA Medical Center, Palo Alto, CA). After mechanical testing, the same  
5 specimens were dehydrated in graded ethanols, embedded in methyl methacrylate, and cut with a low speed bone saw (Isomet, Buehler, Lake Bluff, IL) to  $\approx 100 \mu\text{m}$  in thickness. The cross-sections (2-3) from the femoral proximal midshafts were glued on plastic slides, ground  
10 and polished to  $\approx 40 \mu\text{m}$  thickness and analyzed under polarized light for cortical bone internal structure. The measurements included thickness of the inner and outer lamellar bone layer and thickness of the medially placed woven bone. Following these measurements, samples  
15 were stained by the von Kossa method, counterstained with toluidine blue and cover-slipped. Static bone analyses were performed including the calculation of the second moment of inertia parameter.

#### 20 4. Cellular Bone Analyses

Right tibias were fixed in 10% neutral buffered formalin for 48h, demineralized in Cal-Rite decalcifying solution (Richland-Allan Medical, Richland, MI) for two weeks and processed for paraffin embedding. Three  $\mu\text{m}$   
25 thick longitudinal sections through the proximal tibial metaphysis were stained with hematoxylin and eosin and Gomori's trichrome and were used for cellular bone analyses.

#### 30 5. Statistical Analyses

Differences between plasma glucose values at zero and two hours in Table 3 were analyzed by Student's t-test. A two-factor analysis of variance was employed in Table 3 for comparing differences between treatment  
35 groups. Differences between the groups shown in Tables



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7-16 or Figures 2a-b, 3, 4, 7a-d were tested for significance in a one-way analysis of variance. When the analysis of variance indicated significant differences among means, the differences were evaluated using  
5 Dunnett's t-test and Fisher's Protected Least Significant Difference method for multiple comparisons (Netter et al. (1982) "Applied Statistics," Allyn and Bacon, Boston.) Statistical significance was considered at  $P < 0.05$ , and results were expressed as the mean  $\pm$  standard error (SE).

10 c. Results

Analysis of data revealed site-specific alterations of cortical and cancellous bone to ovariectomy and treatment with rhIGF-I and rhIGF-I/IGFBP-  
15 3 complex. As shown in Table 3, the 2.5 mg/kg dose of rhIGF-I caused acute hypoglycemia which did not occur with any of the doses of rhIGF-I/IGFBP-3. The data presented in Table 4 indicate that no treatment had any influence on glycosylated hemoglobin values and that  
20 increasing doses of rhIGF-I and rhIGFBP-3 led to higher serum levels of total IGF-I, rhIGF-I and rhIGFBP-3. Combining rhIGF-I with rhIGFBP-3 resulted in higher serum values of these agents than observed in rats treated with only rhIGF-I.

25 Spine BMD data throughout the study along with means of the individual changes are presented in Table 4A. As expected, ovariectomy led to a decline in spine BMD which was prevented by the 7.5 mg/kg dose of  
30 rhIGF-I/IGFBP-3. Some protection was observed with the two lower doses of rhIGF-I/IGFBP-3 but not with the two doses of rhIGF-I. The DEXA data obtained on excised tibia (Table 5) and femurs (Table 6) show dose-related increases in global BMC and global BMD for both rhIGF-I  
35 and rhIGF-I/IGFBP-3.

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### 1. Cortical Bone

Treatment with both formulations tested caused a dose dependent increase in the body weight and in the lean body mass of treated rats (Figs. 2A and 2B).

5 Periosteal bone formation activity was higher in all treated groups and at all sites measured relative to Sham or OVX controls (Tables 7, 8, 9, 11). However, the group of rats treated with 7.5 mg/kg of rhIGF-I/IGFBP-3 complex exhibited the highest increase in the periosteal bone  
10 formation rate parameter (Figs. 4A and 5C, 5CC).

Endocortical bone resorption in treated rats, which is a hallmark of cortical bone changes in estrogen deficient women and rats, were not different from values obtained for the Sham-op controls (Fig. 4B), showing  
15 significantly lower values compared to OVX rats at the femoral neck location (Table 11). Also, bone formation at the endocortical envelope was increased at both bone sites measured (femoral diaphyses, Table 8).

Internal structural analyses showed normal  
20 "lamellar" configuration of the bone formed on both cortical envelopes after rhIGF-I/IGFBP-3 treatment (Table 9; Fig. 6). Increased modeling-dependent bone formation on the periosteal and endocortical bone envelopes and moderate or decreased bone resorption on the endocortical  
25 envelope resulted in thicker cortical bone in the treated rats (Table 7, 9, 11; Figs. 5 and 6). Increased cortical thickness and newly formed lamellar bone which is mechanically superior to woven bone resulted in the higher failure torque and polar moment of inertia  
30 parameters in rats treated with 7.5 mg/kg of rhIGF-I/IGFBP-3 complex (Table 10).

All treated animals exhibited similar increases in longitudinal bone growth when compared to OVX of Sham rats (Fig. 3). In general, treatment with rhIGF-I/  
35 IGFBP-3 caused cortical bone thickening by adding

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"lamellar" bone on both envelopes. The increase in lean body component might potentiate periosteal bone formation by increasing the muscle pull on the periosteum. Both "lamellar" structure and increased cortical thickness improved cortical bone strength after rhIGF-I/IGFBP-3 treatment.

## 2. Cancellous Bone

Cancellous bone dynamics and structure were examined at four different locations in the rat skeleton. Differences in different locations in the bone turnover rates and functional anatomy between the designated sites (distal femoral epiphysis, distal femoral metaphysis, femoral mid-neck, and lumbar vertebral bodies) were measured. Treatment with rhIGF-I/IGFBP-3 increased bone formation parameters at all four sites (Tables 12, 13, 14, 15; Figs. 7 and 8). The increase in bone formation rates (volume referents; Figs. 7A-7D) was the highest in the group treated with 7.5 mg/kg of rhIGF-I/IGFBP-3 complex. Bone resorption had similar or lower values compared to OVX rats (Tables 12-15). Such turnover activity resulted in increased trabecular thickness and preservation of the trabecular number in treated rats at all sites measured, except in the femoral metaphysis.

Primary and secondary spongiosa at the femoral metaphyseal site was highly affected by the endochondral bone elongation, particularly in the OVX rats and after treatment with rhIBF or rhIBF-I/IBFBP-3 (Figs. 9A-9C). This bone site has the highest bone turnover rate. OvX caused disappearance of "metabolic" trabeculae in the central metaphyseal region with no mechanical value (Fig. 9B) while treatment with rhIGF-I/IGFBP-3 complex helped the restoration (Fig. 9C).

Structural trabecular bone analyses were performed at the femoral neck location, which is the most

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relevant bone site for fractures in humans (Table 16; Figs. 10A-10C). Besides having a very unique anatomy, this bone site combines cortical and cancellous bone interconnected at the endocortical surface. Cortical  
5 bone thickness, cancellous bone mass, structure, and connectivity between endocortical surface and trabecular network are all important determinants of the bone strength in the femoral neck region. The treatment with the rhIGF-I/IGFBP-3 improved trabecular bone structure  
10 and connectivity between the trabecular network and endocortical surface. In general, data presented here revealed benefits of systemically administered rhIBF-I/IBFBP-3 on the entire musculo-skeletal system in  
15 previously ovariectomized rats.

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Table 7: Cortical bone morphometry measured at the ilio-fibular junction in rats treated with rhIGF-I alone, or with equimolar doses of rhIGF-I/IGFBP-3 complex for eight weeks.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)				rhIGF-I (mg/kg)+rhIGFBP-3			
			0.9	2.6	0.9	2.6	0.9	2.6	0.9	2.6
Periosteal										
perimeter (mm)	8.64±0.12	9.24±0.15†	9.19±0.18†	9.29±0.27†	9.21±0.21†	9.43±0.17†	9.21±0.21†	9.43±0.17†	9.21±0.21†	9.55±0.17†
Endocortical										
perimeter (mm)	3.56±0.06	4.03±0.18†	3.8±0.15	4.12±0.2†	3.73±0.14	3.69±0.09b	3.73±0.14	3.69±0.09b	3.73±0.14	3.71±0.11†
area (mm <sup>2</sup> )	5.52±0.09	5.69±0.18†	5.59±0.27	5.83±0.24†	5.63±0.18	5.78±0.14*	5.63±0.18	5.78±0.14*	5.63±0.18	6.27±0.16†
Marrow										
area (mm <sup>2</sup> )	0.95±0.02	1.08±0.05†	0.92±0.09	1.03±0.06†	0.67±0.05†	0.81±0.05*	0.67±0.05†	0.81±0.05*	0.67±0.05†	0.93±0.04*
Cortical										
area (mm <sup>2</sup> )	4.57±0.07	4.62±0.13†	4.68±0.29*	4.8±0.17†	4.95±0.14†	4.97±0.13†	4.95±0.14†	4.97±0.13†	4.95±0.14†	5.34±0.14†
Percent periosteal double labeled surface (%)	11.76±4.6	10.33±2.9†	43.09±8.4†	67.27±9.6†	44.12±3.8†	73.09±4.5†	44.12±3.8†	73.09±4.5†	44.12±3.8†	90.04±2.2†
Periosteal new bone area (μm <sup>2</sup> )	0.004±0.002	0.002±0.001†	0.02±0.01†	0.03±0.01†	0.02±0.004†	0.07±0.01†.b	0.02±0.004†	0.07±0.01†.b	0.02±0.004†	0.09±0.004†
Periosteal mineralized surface (%)	12.16±4.7	8.33±3.2†	43.52±8.8†	52.29±9.0†	44.68±4.1†	63.65±5.5†	44.68±4.1†	63.65±5.5†	44.68±4.1†	84.26±2.8†
Periosteal mineral appositional rate (μm/d)	0.33±0.17	0.17±0.12†	0.71±0.05†	1.03±0.09†	0.62±0.04†	1.42±0.1†.b	0.62±0.04†	1.42±0.1†.b	0.62±0.04†	1.43±0.02†
Periosteal bone formation rate (μm <sup>2</sup> /μm/d)	0.06±0.03	0.02±0.02	0.27±0.08†	0.4±0.11†	0.21±0.04†	0.77±0.11†.b	0.21±0.04†	0.77±0.11†.b	0.21±0.04†	1.09±0.06†
Periosteal bone formation rate (%/y)	5.22±2.18	1.63±3.65	18.39±14.2†	28.8±8.78†	13.67±6.8†	53.7±13.8†.b	13.67±6.8†	53.7±13.8†.b	13.67±6.8†	72.6±12.5†

Data are expressed as the mean ± SE; † Significantly different from Sham group, P<0.05 by Dunnett's test; \* Significantly different from Ovx group, P<0.05 by Dunnett's test; b Significantly different from rhIGF-I (2.6 mg/kg) group, P<0.05 by Fisher's PLSD test.

**Table 8:** Dynamic cortical bone parameters measured at the femoral diaphyses in control rats and rats treated with 7.5mg/kg of rhIGF-I/IGFBP-3 complex for eight weeks.

Parameter	Basal Sham	Basal Ovx	Sham	Ovx	IGF-I/IGFBP-3
Periosteal mineralizing surface (%)	50.18±5.26	58.34±3.15	41.38±3.66 <sup>b</sup>	32.87±4.05 <sup>a,b</sup>	86.45±4.72 <sup>a,b,c,d</sup>
Periosteal mineral appositional rate (µm/d)	0.61±0.02	1.03±0.04 <sup>a</sup>	0.76±0.05 <sup>a,b</sup>	0.83±0.04 <sup>a,b</sup>	1.29±0.07 <sup>a,b,c,d</sup>
Periosteal bone formation rate (µm <sup>2</sup> /µm/d)	0.23±0.03	0.49±0.02 <sup>a</sup>	0.26±0.03 <sup>b</sup>	0.2±0.04 <sup>b</sup>	1.07±0.13 <sup>a,b,c,d</sup>
Periosteal bone formation rate (%/y)	21.27±2.5	45.85±0.9 <sup>a</sup>	24.89±1.48 <sup>b</sup>	21.4±3.02 <sup>b</sup>	88.81±10.3 <sup>a,b,c,d</sup>
Endocortical mineralizing surface (%)	1.02±0.43	4.03±0.69	1.69±0.27	2.14±0.4 <sup>c</sup>	17.79±4.22 <sup>a,b,c,d</sup>
Endocortical mineral appositional rate (µm/d)	0.009±0.01	0.425±0.19 <sup>a</sup>	0.068±0.06 <sup>b</sup>	0.052±0.05 <sup>b</sup>	0.807±0.13 <sup>a,b,c,d</sup>
Endocortical bone formation rate (µm <sup>2</sup> /µm/d)	0.008±0.05	0.023±0.01	0.002±0.001	0.001±0.001	0.173±0.046 <sup>a,b,c,d</sup>
Endocortical bone formation rate (%/y)	0.04±0.02	2.16±0.86	0.42±0.42	0.32±0.32	7.79±2.35 <sup>a,b,c,d</sup>
Endocortical resorption surface (%)	1.01±0.25	4.14±0.8 <sup>a</sup>	1.79±0.23 <sup>b</sup>	6.33±0.79 <sup>a,b,c</sup>	2.71±0.33 <sup>a,d</sup>

Data are expressed as the mean ± SE; Significant difference  $P < 0.05$  by Fisher's PLSD test.

a Significantly different from Basal Sham group; b Significantly different from Basal Ovx group;

c Significantly different from Sham group; d Significantly different from Ovx group.

**Table 9:** Static cortical parameters measured at the femoral midshafts, and structural parameters measured at the postero-lateral 1/4 of the cross-sectional areas in control rats and rats treated with 7.5mg/kg of rhIGF-I/IGFBP-3 complex for eight weeks.

<u>Parameter</u>	<u>Basal Sham</u>	<u>Basal Qvx</u>	<u>Sham</u>	<u>Ovx</u>	<u>IGF-I/IGFBP-3</u>
Periosteal					
perimeter (mm)	9.54±0.09	9.65±0.23	9.76±0.11	10.51±0.19 <sup>a,b,c</sup>	11.61±0.41 <sup>a,b,c,d</sup>
Endocortical					
perimeter (mm)	5.4±0.13	5.79±0.18	5.38±0.12	5.98±0.18 <sup>a,c</sup>	5.69±0.15
Organ					
area (mm <sup>2</sup> )	6.02±0.13	5.89±0.22	6.05±0.16	6.65±0.19 <sup>a,b,c</sup>	7.03±0.15 <sup>a,b,c</sup>
Marrow					
area (mm <sup>2</sup> )	1.76±0.05	2.0±0.13	1.82±0.09	2.18±0.13 <sup>a,c</sup>	1.86±0.09 <sup>d</sup>
Cortical					
area (mm <sup>2</sup> )	4.26±0.09	3.89±0.1	4.23±0.12	4.46±0.09 <sup>b</sup>	5.17±0.09 <sup>a,b,c,d</sup>
Average cortical					
width (μm)	601.4±7.9	568.6±1.5 <sup>a</sup>	597.5±6.1 <sup>b</sup>	574.8±3.8 <sup>a,c</sup>	614.8±5.6 <sup>b,c,d</sup>
Percent cortical					
area (%)	70.7±0.5	69.16±0.77	69.9±1.1	67.3±1.2 <sup>a</sup>	73.6±0.9 <sup>a,b,c,d</sup>
<u>Structural parameters:</u>					
Outer lamellar					
layer (μm)	277.1±8.9	273.5±8.4	274.5±11.3	316.8±14.6 <sup>a,b,c</sup>	280.3±10.1 <sup>d</sup>
Central woven					
layer (μm)	119.8±5.1	114.6±6.0	120.5±6.4	103.7±14.5	114.2±6.2
Inner lamellar					
layer (μm)	213.3±8.1	145.4±11.2 <sup>a</sup>	202.6±9.2 <sup>b</sup>	70.4±8.8 <sup>a,b,c</sup>	242.6±13.7 <sup>a,b,c,d</sup>

Data are expressed as the mean ± SE; Significant difference  $P < 0.05$  by Fisher's PLSD test.

<sup>a</sup> Significantly different from Basal Sham group; <sup>b</sup> Significantly different from Basal Ovx group;

<sup>c</sup> Significantly different from Sham group; <sup>d</sup> Significantly different from Ovx group.

**Table 10:** Femoral failure strength and general parameters in control rats and rats treated with 7.5mg/kg of rhIGF-I/IGFBP-3 complex for eight weeks.

<u>Parameter</u>	<u>Basal Sham</u>	<u>Basal Ovx</u>	<u>Sham</u>	<u>Ovx</u>	<u>IGF-I/IGFBP-3</u>
Failure torque (N/mm)	461.9±526.8	387.0±41.1 <sup>a</sup>	390.6±31.2	443.6±21.2 <sup>b,c</sup>	471.5±15.3 <sup>b,c,d</sup>
Torsional rigidity (Ncm <sup>2</sup> /rad)	454±58	439±66	468±98	479±93	468±39
Linear stiffness (N-m/degrees)	43.4±2.6	40.7±2.5	42.6±2.5	43.1±3.9	44.9±2.8
Polar moment of inertia (mm <sup>4</sup> )	6.6±0.7	6.3±0.9	6.5±0.6	6.8±1.3 <sup>b</sup>	9.3±1.8 <sup>a,b,d</sup>
Femoral length (mm)	36.0±0.3	35.8±0.3	36.6±0.4	36.8±0.4 <sup>b</sup>	37.9±0.2 <sup>a,b,c,d</sup>
Femoral area (cm <sup>2</sup> )	1.67±0.03	1.66±0.04	1.73±0.03	1.74±0.04	1.86±0.02 <sup>a,b,c,d</sup>
Femoral BMC (g)	0.41±0.01	0.37±0.01 <sup>a</sup>	0.44±0.02 <sup>b</sup>	0.34±0.01 <sup>c</sup>	0.45±0.01 <sup>a,b,d</sup>
Femoral BMD (g/cm <sup>2</sup> )	0.25±0.003	0.23±0.004 <sup>a</sup>	0.25±0.01 <sup>b</sup>	0.23±0.003 <sup>a,c</sup>	0.24±0.002 <sup>b,d</sup>

Data are expressed as the mean ± SE; Significant difference  $P < 0.05$  by Fisher's PLSD test.

<sup>a</sup> Significantly different from Basal Sham group; <sup>b</sup> Significantly different from Basal Ovx group;

<sup>c</sup> Significantly different from Sham group; <sup>d</sup> Significantly different from Ovx group.



**Table 11:** Cortical bone morphometry measured at femoral neck cross-sectional areas after eight weeks of treatment with rhIGF-I alone or rhIGF-I/IGFBP-3 complex.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)		rhIGF-I (mg/kg)+equimolar rhIGFBP-3	
			0.9	2.6	0.9	2.6
<i>Entire organ</i>						
Cortical bone						
area (%)	83.3±1.4	78.9±1.7*	84.3±1.0 <sup>a</sup>	84.9±1.3 <sup>a</sup>	84.1±1.5 <sup>a</sup>	84.7±2.2 <sup>a</sup>
Bone marrow						87.3±1.4 <sup>a</sup>
area (%)	13.9±0.1	13.1±0.1*	8.4±0.1 <sup>*,a</sup>	6.4±0.1 <sup>*,a</sup>	7.8±0.1 <sup>*,a</sup>	7.9±0.1 <sup>*,a</sup>
Average cortical						3.3±0.02 <sup>a,c</sup>
with (μm)	472±9	447±8	465±11	471±12	453±12	466±14
<i>Periosteal envelope</i>						
Mineralized						497±8 <sup>*,a,c</sup>
surface (%)	4.2±0.4	7.1±1.3*	7.9±1.4*	11.8±1.0 <sup>*,a</sup>	8.0±0.9*	9.7±1.7*
Mineral appositional						15.4±1.3 <sup>*,a,c</sup>
rate (μm/d)	0.24±0.01	0.24±0.01	0.38±0.03 <sup>*,a</sup>	0.48±0.02 <sup>*,a</sup>	0.36±0.01 <sup>*,a</sup>	0.35±0.01 <sup>*,a</sup>
Bone formation						0.48±0.02 <sup>*,a,b</sup>
rate (μm <sup>2</sup> /μm/d)	0.01±0.001	0.01±0.003	0.02±0.01*	0.04±0.01 <sup>*,a</sup>	0.02±0.003*	0.02±0.01 <sup>*,b</sup>
Bone formation						0.05±0.01 <sup>*,a,c</sup>
rate (%/y)	7.3±0.8	9.6±1.3	34.7±5.2 <sup>*,a</sup>	55.4±4.5 <sup>*,a</sup>	37.47±6.1 <sup>*,a</sup>	37.0±5.8 <sup>*,a,b</sup>
						59.0±6.2 <sup>†,a,c</sup>

**Table 11 (cont'd):** Cortical bone morphometry measured at femoral neck cross-sectional areas after eight weeks of treatment with rhIGF-I alone or rhIGF-I/IGFBP-3 complex.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)		rhIGF-I (mg/kg)+equimolar rhIGFBP-3	
			0.9	2.6	0.9	2.6
Endocortical envelope						
Mineralized surface (%)	6.5±0.6	8.8±1.3	12.2±1.5 <sup>a</sup>	11.7±1.6 <sup>a</sup>	19.7±0.9 <sup>a,b</sup>	17.9±2.3 <sup>a,b</sup>
Mineral appositional rate (µm/d)	0.23±0.01	0.24±0.01	0.49±0.02 <sup>a</sup>	0.49±0.03 <sup>a</sup>	0.6±0.01 <sup>a</sup>	0.53±0.03 <sup>a</sup>
Bone formation rate (µm <sup>2</sup> /µm/d)	0.01±0.001	0.01±0.002	0.05±0.02 <sup>a</sup>	0.04±0.01 <sup>a</sup>	0.07±0.01 <sup>a</sup>	0.08±0.01 <sup>a,b</sup>
Bone formation rate (%/y)	3.2±0.3	5.8±1.1	27.8±4.7 <sup>a</sup>	27.4±5.4 <sup>a</sup>	46.7±6.9 <sup>a</sup>	60.6±11.1 <sup>a,b</sup>
Resorption perimeter (%)	4.8±0.2	7.5±0.6 <sup>a</sup>	5.2±0.6 <sup>a</sup>	3.9±0.4 <sup>a</sup>	4.8±0.4	3.9±0.5 <sup>a,b</sup>
						3.2±0.3 <sup>a,c</sup>

Data are expressed as the mean ± SE; <sup>a</sup>P<0.05 Significantly different from Sham group; <sup>b</sup>P<0.05 Significantly different from OvX group; <sup>a</sup>P<0.05 Significantly different from rhIGF-I (0.9 mg/kg) group; <sup>b</sup>P<0.05 Significantly different from rhIGF-I (2.6 mg/kg) group; <sup>c</sup>P<0.05 Significantly different from rhIGF-I/IGFBP-3 (0.9 mg/kg and/or 2.6 mg/kg) group.

Table 12: Cancellous bone morphometry measured at distal femoral metaphysis after eight weeks of treatment with rhIGF-I alone or rhIGF-I/IGFBP-3 complex.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)		rhIGF-I (mg/kg)+equimolar rhIGFBP-3	
			0.9	2.6	0.9	2.6
Percent double labeled perimeter (%)	6.05±0.64	17.41±1.56†	14.21±0.77†	17.32±1.33†	18.02±0.84†	16.23±1.11†
Percent resorption perimeter (%)	5.89±0.3	11.01±1.46†	9.61±0.67†	14.01±2.07†	9.08±1.01†	10.16±0.56† <sup>b</sup>
Corrected mineral appositional rate (µm/d)	0.17±0.03	0.47±0.06†	0.38±0.03†	0.41±0.03†	0.44±0.02†	0.44±0.03†
Mineralized surface (%)	2.52±0.36	8.54±1.5†	5.35±1.79† <sup>*</sup>	6.35±2.44†	10.48±3.24† <sup>*</sup>	13.56±3.53†
Bone formation rate (µm <sup>2</sup> /µm/d)	0.001±0.001	0.021±0.006†	0.014±0.001†	0.012±0.002†	0.015±0.002†	0.014±0.002†
Endochondral growth (µm/d)	3.77±0.21	3.81±0.29	5.57±0.33† <sup>*</sup>	6.44±0.4† <sup>*</sup>	5.89±1.33† <sup>*</sup>	5.89±0.26† <sup>*</sup>
Percent trabecular area (%)	25.02±1.58	14.95±0.92†	19.48±0.78† <sup>*</sup>	14.73±1.33†	16.15±0.99† <sup>a</sup>	17.86±1.41†
Trabecular thickness (µm)	52.35±2.44	58.8±1.29†	66.24±0.58†	56.37±1.88	55.11±1.59	68.2±1.2† <sup>*,b</sup>
number (No/mm) Trabecular	4.78±0.21	2.53±0.12†	2.97±0.1† <sup>*</sup>	2.54±0.17†	2.85±0.13†	2.74±0.13†
separation (µm) Perimeter/Area ratio (mm/mm <sup>2</sup> )	229.8±12.7	492.69±26.2†	393.9±16.9† <sup>*</sup>	501.1±39.2†	429.8±22.4†	447.3±37.1†
	32.43±1.54	28.48±0.64†	25.38±0.37† <sup>*</sup>	29.21±1.0	29.7±0.89 <sup>a</sup>	25.9±0.8† <sup>*,b</sup>
						33.23±0.83

Data are expressed as the mean ± SE; † Significantly different from Sham group, P<0.05 by analysis of variance followed by Dunnett's test;

\* Significantly different from Ovx group, P<0.05 by analysis of variance followed by Dunnett's test;

<sup>a</sup> Significantly different from rhIGF-I (0.9 mg/kg) group, P<0.05 by analysis of variance followed by Fisher's PLSD method;

<sup>b</sup> Significantly different from rhIGF-I (2.6 mg/kg) group, P<0.05 by analysis of variance followed by Fisher's PLSD method.

**Table 13:** Cancellous bone morphometry measured at distal femoral epiphysis after eight weeks of treatment with rhIGF-I alone or rhIGF-I/IGFBP-3 complex.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)			rhIGF-I (mg/kg)+equimolar rhIGFBP-3		
			0.9	2.6	0.9	2.6	0.9	2.6
Percent double labeled perimeter (%)	6.07±0.49	18.72±2.02†	9.35±0.62†*	13.43±1.45†*	22.89±1.32†.a	24.12±2.06†.b	28.24±1.36†*	28.24±1.36†*
Percent resorption perimeter (%)	5.93±0.48	7.18±0.57	5.11±0.39*	5.26±0.54*	4.73±0.49*	5.19±0.33*	7.13±0.52	7.13±0.52
Corrected mineral appositional rate (µm/d)	0.17±0.05	0.51±0.04†	0.57±0.01†	0.39±0.04†*	0.55±0.02†	0.58±0.07†.b	0.7±0.02†*	0.7±0.02†*
Mineralized surface (%)	1.56±0.09	9.65±2.58†	6.01±2.12†*	9.28±3.47†	12.31±3.2†.a	17.71±4.05†.b	21.39±3.87†*	21.39±3.87†*
Bone formation rate (µm <sup>2</sup> /µm/d)	0.001±0.003	0.016±0.004†	0.008±0.001†	0.008±0.002†	0.016±0.003†	0.023±0.005†.b	0.035±0.003†*	0.035±0.003†*
Percent trabecular area (%)	37.19±1.44	31.01±0.74†	41.28±2.06*	37.8±1.67*	36.3±1.54*.a	34.11±0.69	40.11±1.73*	40.11±1.73*
Trabecular thickness (µm)	83.9±2.6	82.68±3.0	92.43±4.13	96.5±2.87†*	92.04±2.33†*	92.01±0.98†*	98.79±4.13†*	98.79±4.13†*
Trabecular number (No/mm)	4.33±0.16	3.79±0.16†	4.45±0.14*	3.71±0.13†	4.42±0.2*	3.89±0.1	4.07±0.17	4.07±0.17
Trabecular separation (µm)	206.4±11.8	265.5±11.6†	191.9±11.9*	241.1±10.9†	207.1±11.9*	240.0±5.7†*	210.9±10.9*	210.9±10.9*
Perimeter/Area ratio (mm/mm <sup>2</sup> )	20.03±0.61	20.43±0.89	18.2±0.76	16.52±0.97†*	19.17±1.15	18.45±0.41	17.06±0.84†*	17.06±0.84†*

Data are expressed as the mean ± SE; † Significantly different from Sham group, P<0.05 by analysis of variance followed by Dunnett's test;

\* Significantly different from Ovx group, P<0.05 by analysis of variance followed by Dunnett's test;

a Significantly different from rhIGF-I (0.9 mg/kg) group, P<0.05 by analysis of variance followed by Fisher's PLSD method;

b Significantly different from rhIGF-I (2.6 mg/kg) group, P<0.05 by analysis of variance followed by Fisher's PLSD method.

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**Table 14:** Cancellous bone morphometry measured at lumbar vertebral bodies (L4-L5) after eight weeks of treatment with rhIGF-I alone or rhIGF-I/IGFBP-3 complex.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)		rhIGF-I (mg/kg)+equimolar rhIGFBP-3	
			0.9	2.6	0.9	2.6
Percent double labeled perimeter (%)	8.17±0.99	14.2±1.16†	11.23±0.68	14.31±1.47†	12.0±0.79†	14.12±1.3†
Percent resorption perimeter (%)	6.19±0.48	8.88±0.97†	5.37±0.59*	10.1±1.26†	6.46±0.57	11.34±0.99†
Corrected mineral appositional rate (µm/d)	0.13±0.04	0.33±0.04†	0.4±0.01†	0.32±0.03†	0.29±0.03†,a	0.35±0.02†
Mineralized surface (%)	2.17±0.84	7.38±2.46†	6.28±1.25†	7.86±1.68†	7.21±2.15†	8.56±2.03†
Bone formation rate (µm <sup>2</sup> /µm/d)	0.001±0.001	0.008±0.002†	0.009±0.001†	0.007±0.002†	0.011±0.001†	0.007±0.002†
Percent trabecular area (%)	36.53±1.89	28.46±1.32†	30.06±1.32†	35.04±0.97*	32.95±1.46*	34.11±2.99*
Trabecular thickness (µm)	57.73±2.56	57.26±1.58	58.62±0.99	65.87±3.01†,*	63.66±3.34†,*	64.44±3.5†,*
Trabecular number (No/mm)	6.32±0.09	4.96±0.17†	5.12±0.18†	5.37±0.13*	5.2±0.15	5.25±0.24
Trabecular separation (µm)	145.1±6.05	210.2±11.1†	198.8±10.4†,*	174.8±3.15*	186.2±6.7*	185.0±15.0*
Perimeter/Area ratio (mm/mm <sup>2</sup> )	29.51±0.36	29.32±0.84	28.51±0.48	25.69±1.16†,*	26.74±1.46	26.46±1.52
						25.39±1.05†,*

Data are expressed as the mean ± SE; † Significantly different from Sham group, P<0.05 by analysis of variance followed by Dunnett's test;

\* Significantly different from Ovx group, P<0.05 by analysis of variance followed by Dunnett's test;

a Significantly different from rhIGF-I (0.9 mg/kg) group, P<0.05 by analysis of variance followed by Fisher's PLSD method;

b Significantly different from rhIGF-I (2.6 mg/kg) group, P<0.05 by analysis of variance followed by Fisher's PLSD method.

**Table 15:** Cancellous bone morphometry measured at femoral neck cross-sectional areas after eight weeks of treatment with rhIGF-I alone or rhIGF-I/IGFBP-3 complex.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)		rhIGF-I (mg/kg)+equimolar rhIGFBP-3	
			0.9	2.6	0.9	2.6
Percent resorption perimeter (%)	4.5±0.2	10.1±0.8*	6.1±0.5 <sup>a</sup>	5.2±0.4 <sup>a</sup>	6.1±0.6 <sup>a</sup>	6.9±0.7*, <sup>a</sup>
Corrected mineral appositional rate (µm/d)	0.23±0.01	0.32±0.01*	0.52±0.04*, <sup>a</sup>	0.54±0.01*, <sup>a</sup>	0.5±0.04*, <sup>a</sup>	0.52±0.02*, <sup>a</sup>
Mineralized surface (%)	5.8±0.7	11.9±1.3*	12.3±0.9*	13.8±0.9*	14.9±1.4*	15.9±2.6*
Bone formation rate (µm <sup>2</sup> /µm/d)	0.001±0.001	0.006±0.002	0.012±0.002*	0.014±0.003*	0.014±0.003*	0.014±0.002*
Percent trabecular area (%)	12.8±1.9	8.1±0.6*	7.4±1.1*	8.6±1.3*	8.1±1.3*	7.4±0.7*
Trabecular thickness (µm)	83.5±6.2	69.5±5.4	79.9±2.6	75.1±3.8	69.3±8.6	78.3±2.2
Trabecular number (No/mm)	10.8±1.4	5.6±0.5*	6.9±0.9*	8.1±1.4	7.8±0.6*	5.3±0.6*
Separation (µm)	119.4±12.6	263.3±17.1*	206.8±15.2*, <sup>a</sup>	188.3±24.6*, <sup>a</sup>	183.1±20.3*, <sup>a</sup>	186.1±10.3*, <sup>a</sup>
Perimeter/Area ratio (mm/mm <sup>2</sup> )	15.7±1.6	23.9±2.5*	19.6±0.8	20.7±1.0	20.0±2.2	18.3±1.3
						21.9±2.6

Data are expressed as the mean ± SE; \*P<0.05 Significantly different from Sham group; <sup>a</sup>P<0.05 Significantly different from Ovx group; <sup>b</sup>P<0.05 Significantly different from rhIGF-I (0.9 mg/kg) group; <sup>c</sup>P<0.05 Significantly different from rhIGF-I (2.6 mg/kg) group; <sup>d</sup>P<0.05 Significantly different from rhIGF-I/IGFBP-3 (0.9 mg/kg and/or 2.6 mg/kg) group.

**Table 16:** Structural analyses of cancellous bone performed on von Kossa stained cross-sections of the femoral neck from rats treated for 8 weeks with rhIGF-I alone or the rhIGF-I/IGFBP-3 complex. A nodal analysis was employed for trabecular and endocortico-trabecular connectivities and star volume analysis for trabecular separation.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)		rhIGF-I (mg/kg)+equimolar rhIGFBP-3	
			0.9	2.6	0.9	2.6
<i>Nodal analysis</i>						
Number of nodes	12.9±1.7	6.1±0.5*	5.5±0.3*	5.9±0.3*	7.0±0.7*	6.5±0.3*
Number of struts	16.0±1.3	9.9±0.4*	7.3±0.4*, <sup>a</sup>	8.5±0.6*	8.3±0.7*	9.3±0.6*
<i>Type of strut (% of total)</i>						
Free-free	5.8±1.2	20.6±1.8*	5.3±2.6 <sup>a</sup>	8.7±2.9 <sup>a</sup>	6.8±1.9 <sup>a</sup>	8.4±2.5 <sup>a</sup>
Node-free	18.9±1.7	39.6±2.2*	29.8±3.6*	31.3±2.5*	21.2±5.4 <sup>a,b</sup>	24.3±1.2 <sup>a</sup>
Node-node	75.4±2.0	39.7±3.1*	64.9±4.8*, <sup>a</sup>	61.7±3.1*, <sup>a</sup>	68.6±6.2 <sup>a</sup>	66.4±2.4 <sup>a</sup>
<i>Endocortico-trabecular connectivity</i>						
Total number of endocortico-trabecular connections	11.6±0.9	6.8±0.5*	6.0±0.5*	6.4±0.5*	7.8±0.7*	7.6±0.9*
Endocortico-endocortical (%)	95.1±1.2	42.3±1.4	50.2±2.4*	53.2±2.3*, <sup>a</sup>	54.3±1.7*, <sup>a</sup>	55.1±2.3*, <sup>a</sup>
Endocortico-free ending (%)	4.9±0.3	57.7±1.4	49.8±2.1*	46.8±2.0*, <sup>a</sup>	45.7±1.3*, <sup>a</sup>	44.9±2.1*, <sup>a</sup>
<i>Star volume analysis</i>						
Marrow volume (mm <sup>3</sup> )	0.01±0.001	0.03±0.004*	0.01±0.002 <sup>a</sup>	0.013±0.005 <sup>a</sup>	0.01±0.002 <sup>a</sup>	0.006±0.001 <sup>a</sup>

Data are expressed as the mean ± SE; \*P<0.05 Significantly different from Sham group; <sup>a</sup>P<0.05 Significantly different from Ovx group; <sup>a</sup>P<0.05 Significantly different from rhIGF-I (0.9 mg/kg) group; <sup>b</sup>P<0.05 Significantly different from rhIGF-I (2.6 mg/kg) group; <sup>c</sup>P<0.05 Significantly different from rhIGF-I/IGFBP-3 (0.9 mg/kg and/or 2.6 mg/kg) group.

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Example 3Recombinant synthesis of IGF-I

Recombinant rhIGF-I was produced using a gene insert coding for the sequence shown in Figure 11 (SEQ ID NO:1), as detailed below.

Materials

The bacterial strain used is a derivative of Escherichia coli K-12 strain W3110 which has been lysogenized with DE3. (Studier, F. and Moffat, B. [1986] J. Mol. Biol. 189: 113-130). This lysogen carries the gene for T7 RNA polymerase under the control of the lacUV5 promoter.

This host strain was transformed with plasmids pER10088 by selection for tetracycline-resistance.

Description of Plasmids

The three expression vectors used in this work are similar to pJU1002 and pJU1003 (Squires, C.H., et al. [1988] J. Biol. Chem. 263: 16297-16302) except that the genes inserted downstream of the translational coupler are ubiquitin-IGF (pER10088). In addition, pER 10088 differs from pJU1003 in that it does not contain the synthetic 16 bp adaptor sequence at the 5' end of the tet gene in that plasmid; however, it does contain DNA insertions at the unique PvuII site in the pBR322-derived backbone: pER10088 contains a linker 5'...CCTCGAGG...3' at that location.

Description of Gene Inserts

As produced for studies carried out in support of the present invention, the vector pER10088 contained an open reading frame (ORF) comprising (in order 5' to 3') an ATG triplet (initiation), the 76 codons of yeast ubiquitin, 70 synthetic codons of mature human insulin



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growth factor I (Figure 11), and a termination codon. In this case, the ORF is positioned relative to the translational coupler exactly as described by Squires et al. (above) for fibroblast growth factor.

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#### IGF purification

E. coli cells producing IGF-I were broken open using a Gaulin mill or Microfluidizer in 5 volumes/wt (e.g., 1 l/200 g cell paste) of 50 mM sodium acetate and 1 mM EDTA, pH 5.5 while its temperature was maintained at not higher than 10°C. The lysate was checked for the presence of whole E. coli cells using a light microscope. To the lysate was added one-hundredth volume of a 10% (w/v) solution of polyethyleneimine and then centrifuged at 10,000 for 20 minutes to separate supernatant from pellet. The pellet was re-extracted twice more in 2.5 volumes of 20 mM potassium phosphate, 20 mM DTT and 2 M urea pH 5.8 and centrifuged as above. The pellet was used for further purification steps.

IGF was extracted from the pellet with 5 volumes/wt of 20 mM Tris pH 8.0 containing 6 M urea, 40 mM DTT and 1 mM EDTA and filtered through a Sartorius 0.8  $\mu$  filter. The filtrate was diluted with the same buffer to a final protein concentration of 3 mg/ml, then diluted with 2 volumes of 20 mM Tris and 1 mM EDTA pH 8.0. This mixture was subjected to a DNA removal step using protamine sulfate and Q-Sepharose, according to standard methods. Ubiquitin protein peptidase was added to the mixture to release IGF-I from the ubiquitin IGF-I fusion protein, and IGF-I was refolded overnight at ambient temperature. A typical refolding reaction was carried out at about 1 mg/ml protein concentration, 1.5 to 2 M urea and pH range 8-9 in presence of Tris buffer at 20 to 50 mM and a ratio of DTT/Cystamine of 1. Refolding performed as above yields about 40% of the

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correctly refolded IGF-I compared to the initial amount of IGF-I.

The refolded IGF-I solution was clarified, buffer was exchanged using ultrafiltration system and loaded onto a cation exchange column equilibrated in 50 mM sodium acetate buffer, pH 5.5. Following the loading of the IGF-I solution onto the column, the column was washed with the equilibration buffer and purified IGF-I was then eluted from the column by application of 20 column volumes of gradient starting with equilibration buffer and finishing with either 0.25 or 0.5 M sodium chloride. The appropriate fractions of pure IGF-I were pooled.

Pooled fractions from the cation exchange column were acidified to pH 2.5 with 10% trifluoroacetic acid (TFA). The solution was filtered through a 0.2  $\mu$ m filter and loaded onto a Vydac C-4 column equilibrated in 0.1% TFA in water. The column was developed with a linear gradient of 0 to 40% of acetonitrile in 0.1% TFA. The fractions containing purified IGF were assayed by SDS-PAGE, then pooled and lyophilized.

#### Example 4

##### Recombinant Synthesis of IGFBP-3

Recombinant rhIGFBP-3 was produced using as a gene insert coding for the sequence shown in Figure 12 (SEQ ID NO:2), as detailed below. Figures 13-15 show alternative exemplary gene sequences that can be used in the present invention according to methods standard in the art or analogous to the procedures described below. Figure 13 is a preferred coding sequence used in the experiments herein.

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### Materials

The bacterial strain used in experiments carried out in support of the present invention is a derivative of Escherichia coli K-12 strain W3110 which was lysogenized with DE3. (Studier, F. and Moffat, B. [1986] J. Mol. Biol. 189: 113-130). This lysogen carries the gene for T7 RNA polymerase under the control of the lacUV5 promoter. This host strain was transformed with plasmid pDJ12833 by selection for tetracycline-resistance.

### Description of Plasmids

The three expression vectors used in this work are similar to pJU1002 and pJU1003 (Squires, C.H., et al. [1988] J. Biol. Chem. 263: 16297-16302, incorporated herein by reference) except that the gene was inserted downstream of the translational coupler IGFBP-3 (pDJ12833). In addition, pDJ12833 differs from pJU1003 in that it does not contain the synthetic 16 bp adaptor sequence at the 5' end of the tet gene in that plasmid; however, it does contain DNA insertions at the unique PvuII site in the pBR322-derived backbone: pDJ12833 contains a 385 bp fragment carrying the par locus of pSC101 (Meacock, P.A., and Cohen, S.N. [1980] Cell 20: 529-542).

### Description of Gene Insert

pDJ12833 contains an ORF comprising an ATG triplet followed by the 264 codons of mature human IGFBP-3 (Figure 13). The amino terminal 95 codons were synthetic; the remainder were derived from the natural cDNA for this gene.

In this case, the ORF was positioned relative to the translational coupler exactly as described by Squires et al. ([1988] J. Biol. Chem. 263:16297-16302,

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incorporated herein by reference) for fibroblast growth factor.

#### IGFBP-3 purification

5           The E. coli cells producing IGFBP-3 ("BP-3") were broken open using a Gaulin mill or Microfluidizer in 6 volumes/wt (e.g., 1.2 l/200 g cell paste) of 20 mM Tris-HCl and 5 mM EDTA, pH 8 while its temperature was maintained at not higher than 10°C. The lysate was  
10 checked for the presence of whole E. coli cells by light microscopy and then centrifuged at 10,000 g for 20 minutes to separate supernatant from pellet. The pellet was re-extracted twice more in 3 volumes of 20 mM Tris-HCl pH 8 and centrifuged as above. The resulting pellet  
15 was used for further purification.

          BP-3 was extracted from the pellet with 2.5 volumes/wt of 20 mM Tris pH 8.0 containing 6 M GdHCl, 25 mM DTT and 5 mM EDTA and filtered through a Sartorius 0.8  $\mu$  filter. The filtrate was diluted with the above  
20 buffer to a final protein concentration of 4 mg/ml followed with 1 volume of 20 mM Tris and 5 mM EDTA pH 8.0. This mixture was subjected to a DNA removal step using protamine sulfate, according to standard procedures known in the art. The protamine sulfate precipitate was  
25 removed by filtration, and BP-3 was subjected to the refolding reaction. A typical refolding reaction was effected at about 0.5 to 1 mg/ml protein concentration, 1.0 to 1.5 M GdHCl at pH 8-9 in the presence of Tris buffer at 20 to 50 mM, having reducing/oxidizing agents  
30 molar ratio of 1. Refolding performed as above results in greater than 60% of the initial amount of BP-3.

          The refolded BP-3 solution was clarified, buffer was exchanged using an ultrafiltration system and loaded onto a cation exchange column equilibrated in 20  
35 mM sodium phosphate buffer, pH 7. Following loading of

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the BP-3 post-refolding solution onto a cation exchange column, the column was washed with the equilibration buffer and purified BP-3 was then eluted from the column by application of 20 column volumes of gradient starting with equilibration buffer and finishing with 0.8 M sodium chloride. The appropriate fractions of BP-3 were pooled.

The salt concentration of the pooled BP-3-containing fractions was increased to 0.6 M with ammonium sulfate, and then loaded onto a hydrophobic interaction chromatography matrix column equilibrated with salt containing acetate/phosphate buffer having a pH between 5 to 6. BP-3 was eluted with a linear ammonium sulfate gradient ending with 0% ammonium sulfate buffered at pH 5-6. The appropriate fractions were then pooled and saved.

The pooled fractions containing BP-3 were brought to 0.1% trifluoroacetic acid (TFA), and the solution was loaded onto a C4 RP-HPLC column equilibrated with 0.1% TFA in water. The column was washed with equilibration buffer and then eluted with a linear gradient of acetonitrile containing 0.1% TFA. The fractions containing BP-3 were pooled and lyophilized.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: CELTRIX PHARMACEUTICALS, INC.
- (ii) TITLE OF INVENTION: IGF/IGFBP COMPLEX FOR PROMOTING BONE FORMATION AND FOR REGULATING BONE REMODELING
- (iii) NUMBER OF SEQUENCES: 7
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: MORRISON & FOERSTER
  - (B) STREET: 755 Page Mill Road
  - (C) CITY: Palo Alto
  - (D) STATE: California
  - (E) COUNTRY: USA
  - (F) ZIP: 94304-1018
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: Patent In Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: New
  - (B) FILING DATE: New
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: PARK, FREDDIE K.
  - (B) REGISTRATION NUMBER: 35,636
  - (C) REFERENCE/DOCKET NUMBER: 2095-2072.40
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (415) 813-5600
  - (B) TELEFAX: (415) 494-0792
  - (C) TELEX: 706141

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 70 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
 

Gly	Pro	Glu	Thr	Leu	Cys	Gly	Ala	Glu	Leu	Val	Asp	Ala	Leu	Gln	Phe
1				5				10						15	
Val	Cys	Gly	Asp	Arg	Gly	Phe	Tyr	Phe	Asn	Lys	Pro	Thr	Gly	Tyr	Gly
			20				25						30		
Ser	Ser	Ser	Arg	Arg	Ala	Pro	Gln	Thr	Gly	Ile	Val	Asp	Glu	Cys	Cys
			35			40						45			
Phe	Arg	Ser	Cys	Asp	Leu	Arg	Arg	Leu	Glu	Met	Tyr	Cys	Ala	Pro	Leu
50					55					60					

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Lys Pro Ala Lys Ser Ala  
65 70

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 264 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 5
- (D) OTHER INFORMATION: /note= "There is natural heterogeneity at this position; glycine can also occur at this position."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Ala Ser Ser Ala Gly Leu Gly Pro Val Val Arg Cys Glu Pro Cys  
1 5 10 15  
Asp Ala Arg Ala Leu Ala Gln Cys Ala Pro Pro Pro Ala Val Cys Ala  
20 25 30  
Glu Leu Val Arg Glu Pro Gly Cys Gly Cys Cys Leu Thr Cys Ala Leu  
35 40 45  
Ser Glu Gly Gln Pro Cys Gly Ile Tyr Thr Glu Arg Cys Gly Ser Gly  
50 55 60  
Leu Arg Cys Gln Pro Ser Pro Asp Glu Ala Arg Pro Leu Gln Ala Leu  
65 70 75 80  
Leu Asp Gly Arg Gly Leu Cys Val Asn Ala Ser Ala Val Ser Arg Leu  
85 90 95  
Arg Ala Tyr Leu Leu Pro Ala Pro Pro Ala Pro Gly Asn Ala Ser Glu  
100 105 110  
Ser Glu Glu Asp Arg Ser Ala Gly Ser Val Glu Ser Pro Ser Val Ser  
115 120 125  
Ser Thr His Arg Val Ser Asp Pro Lys Phe His Pro Leu His Ser Lys  
130 135 140  
Ile Ile Ile Ile Lys Lys Gly His Ala Lys Asp Ser Gln Arg Tyr Lys  
145 150 155 160  
Val Asp Tyr Glu Ser Gln Ser Thr Asp Thr Gln Asn Phe Ser Ser Glu  
165 170 175  
Ser Lys Arg Glu Thr Glu Tyr Gly Pro Cys Arg Arg Glu Met Glu Asp  
180 185 190  
Thr Leu Asn His Leu Lys Phe Leu Asn Val Leu Ser Pro Arg Gly Val  
195 200 205  
His Ile Pro Asn Cys Asp Lys Lys Gly Phe Tyr Lys Lys Lys Gln Cys  
210 215 220  
Arg Pro Ser Lys Gly Arg Lys Arg Gly Phe Cys Trp Cys Val Asp Lys  
225 230 235 240





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TCC AAG CGG GAG ACA GAA TAT GGT CCC TGC CGT AGA GAA ATG GAA GAC	576
Ser Lys Arg Glu Thr Glu Tyr Gly Pro Cys Arg Arg Glu Met Glu Asp	
180 185 190	
ACA CTG AAT CAC CTG AAG TTC CTC AAT GTG CTG AGT CCC AGG GGT GTA	624
Thr Leu Asn His Leu Lys Phe Leu Asn Val Leu Ser Pro Arg Gly Val	
195 200 205	
CAC ATT CCC AAC TGT GAC AAG AAG GGA TTT TAT AAG AAA AAG CAG TGT	672
His Ile Pro Asn Cys Asp Lys Lys Gly Phe Tyr Lys Lys Lys Gln Cys	
210 215 220	
CGC CCT TCC AAA GGC AGG AAG CGG GGC TTC TGC TGG TGT GTG GAT AAG	720
Arg Pro Ser Lys Gly Arg Lys Arg Gly Phe Cys Trp Cys Val Asp Lys	
225 230 235 240	
TAT GGG CAG CCT CTG CCA GGC TAC ACC ACC AAG GGG AAG GAG GAC GTG	768
Tyr Gly Gln Pro Leu Pro Gly Tyr Thr Thr Lys Gly Lys Glu Asp Val	
245 250 255	
CAC TGC TAC AGC ATG CAG AGC AAG TAG	795
His Cys Tyr Ser Met Gln Ser Lys	
260	

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 264 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Gly Ala Ser Ser Ala Gly Leu Gly Pro Val Val Arg Cys Glu Pro	
1 5 10 15	
Cys Asp Ala Arg Ala Leu Ala Gln Cys Ala Pro Pro Ala Val Cys Ala	
20 25 30	
Glu Leu Val Arg Glu Pro Gly Cys Gly Cys Cys Leu Thr Cys Ala Leu	
35 40 45	
Ser Glu Gly Gln Pro Cys Gly Ile Tyr Thr Glu Arg Cys Gly Ser Gly	
50 55 60	
Leu Arg Cys Gln Pro Ser Pro Asp Glu Ala Arg Pro Leu Gln Ala Leu	
65 70 75 80	
Leu Asp Gly Arg Gly Leu Cys Val Asn Ala Ser Ala Val Ser Arg Leu	
85 90 95	
Arg Ala Tyr Leu Leu Pro Ala Pro Pro Ala Pro Gly Asn Ala Ser Glu	
100 105 110	
Ser Glu Glu Asp Arg Ser Ala Gly Ser Val Glu Ser Pro Ser Val Ser	
115 120 125	
Ser Thr His Arg Val Ser Asp Pro Lys Phe His Pro Leu His Ser Lys	
130 135 140	
Ile Ile Ile Ile Lys Lys Gly His Ala Lys Asp Ser Gln Arg Tyr Lys	
145 150 155 160	

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[illegible]

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 811 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGTGCTTCTT	CTGCTGGTCT	TGGACCAGTT	GTTCTGTTGTG	AACCATGTGA	TGCACGAGCT	60
TTAGCTCAAT	GTGCTCCACC	ACCAGCTGTT	TGTGCTGAAT	TAGTTCGAGA	ACCAGGTTGT	120
GGTTGTTGTT	TAACCTGTGC	TTTATCTGAA	GGTCAACCAT	GTGGTATTTA	TACTGAACGT	180
TGCGGTAGTG	GTTTGCGTTG	TCAACCAAGC	CCAGATGAAG	CTAGGCCTTT	ACAAGCATT	240
TTAGATGGTC	GAGGTCTGTG	TGTTAATGCG	TCCGCTGTTT	CTCGATTGCG	CGCTTATTTA	300
TTACCTGCCC	CACCGGCACC	GGGTAACGCC	TCCGAAAGCG	AAGAGGATCG	TTCTGCGGGT	360
TCCGTTGAAT	CTCCAAGTGT	GAGTTCTACC	CATCGAGTTA	GCGACCCGAA	ATTTTCATCCG	420
TTGCACTCTA	AAATCATTAT	TATTAATAAG	GGTCACGCAA	AGGATTCTCA	ACGTTATAAG	480
GTGGATTATG	AAAGCCAATC	TACCGACACT	CAAAATTTTA	GTAGTGAAAG	TAAACGTGAA	540
ACCGAGTACG	GCCCGTGTGC	ACGTGAGATG	GAGGATACCT	TAAACCATTT	AAAATTTTGT	600
AACGTTTTAT	CCCCGCGTGG	CGTTCATATC	CCGAATTGCG	ATAAAAAAGG	CTTCTACAAA	660
AAGAAACAAT	GCCGTCCGAG	TAAGGGTCGT	AAACGAGGTT	TTTGTTGGTG	CGTTGACAAA	720
TACGGTCAAC	CGTTGCCGGG	TTATACTACT	AAAGGCATAAG	AAGATGTTCA	TTGTTATTCT	780
ATGCAATCTA	AATAATGCAT	CTCGAGAATT	C			811

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 876 base pairs

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(B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ix) FEATURE:

(A) NAME/KEY: CDS  
 (B) LOCATION: 1..873

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATG CAG CGG GCG CGA CCC ACG CTC TGG GCC GCT GCG CTG ACT CTG CTG	48
Met Gln Arg Ala Arg Pro Thr Leu Trp Ala Ala Ala Leu Thr Leu Leu	
265 270 275 280	
GTG CTG CTC CGC GGG CCG CCG GTG GCG CGG GCT GGC GCG AGC TCG GCG	96
Val Leu Leu Arg Gly Pro Pro Val Ala Arg Ala Gly Ala Ser Ser Ala	
285 290 295	
GGC TTG GGT CCC GTG GTG CGC TGC GAG CCG TGC GAC GCG CGT GCA CTG	144
Gly Leu Gly Pro Val Val Arg Cys Glu Pro Cys Asp Ala Arg Ala Leu	
300 305 310	
GCC CAG TGC GCG CCT CCG CCC GCC GTG TGC GCG GAG CTG GTG CGC GAG	192
Ala Gln Cys Ala Pro Pro Pro Ala Val Cys Ala Glu Leu Val Arg Glu	
315 320 325	
CCG GGC TGC GGC TGC TGC CTG ACG TGC GCA CTG AGC GAG GGC CAG CCG	240
Pro Gly Cys Gly Cys Cys Leu Thr Cys Ala Leu Ser Glu Gly Gln Pro	
330 335 340	
TGC GGC ATC TAC ACC GAG CGC TGT GGC TCC GGC CTT CGC TGC CAG CCG	288
Cys Gly Ile Tyr Thr Glu Arg Cys Gly Ser Gly Leu Arg Cys Gln Pro	
345 350 355 360	
TCG CCC GAC GAG GCG CGA CCG CTG CAG GCG CTG CTG GAC GGC CGC GGG	336
Ser Pro Asp Glu Ala Arg Pro Leu Gln Ala Leu Leu Asp Gly Arg Gly	
365 370 375	
CTC TGC GTC AAC GCT AGT GCC GTC AGC CGC CTG CGC GCC TAC CTG CTG	384
Leu Cys Val Asn Ala Ser Ala Val Ser Arg Leu Arg Ala Tyr Leu Leu	
380 385 390	
CCA GCG CCG CCA GCT CCA GGA AAT GCT AGT GAG TCG GAG GAA GAC CGC	432
Pro Ala Pro Pro Ala Pro Gly Asn Ala Ser Glu Ser Glu Glu Asp Arg	
395 400 405	
AGC GCC GGC AGT GTG GAG AGC CCG TCC GTC TCC AGC ACG CAC CGG GTG	480
Ser Ala Gly Ser Val Glu Ser Pro Ser Val Ser Ser Thr His Arg Val	
410 415 420	
TCT GAT CCC AAG TTC CAC CCC CTC CAT TCA AAG ATA ATC ATC ATC AAG	528
Ser Asp Pro Lys Phe His Pro Leu His Ser Lys Ile Ile Ile Ile Lys	
425 430 435 440	
AAA GGG CAT GCT AAA GAC AGC CAG CGC TAC AAA GTT GAC TAC GAG TCT	576
Lys Gly His Ala Lys Asp Ser Gln Arg Tyr Lys Val Asp Tyr Glu Ser	
445 450 455	
CAG AGC ACA GAT ACC CAG AAC TTC TCC TCC GAG TCC AAG CGG GAG ACA	624
Gln Ser Thr Asp Thr Gln Asn Phe Ser Ser Glu Ser Lys Arg Glu Thr	
460 465 470	
GAA TAT GGT CCC TGC CGT AGA GAA ATG GAA GAC ACA CTG AAT CAC CTG	672
Glu Tyr Gly Pro Cys Arg Arg Glu Met Glu Asp Thr Leu Asn His Leu	
475 480 485	

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AAG TTC CTC AAT GTG CTG AGT CCC AGG GGT GTA CAC ATT CCC AAC TGT Lys Phe Leu Asn Val Leu Ser Pro Arg Gly Val His Ile Pro Asn Cys 490 495 500	720
GAC AAG AAG GGA TTT TAT AAG AAA AAG CAG TGT CGC CCT TCC AAA GGC Asp Lys Lys Gly Phe Tyr Lys Lys Lys Gln Cys Arg Pro Ser Lys Gly 505 510 515 520	768
AGG AAG CGG GGC TTC TGC TGG TGT GTG GAT AAG TAT GGG CAG CCT CTC Arg Lys Arg Gly Phe Cys Trp Cys Val Asp Lys Tyr Gly Gln Pro Leu 525 530 535	816
CCA GGC TAC ACC ACC AAG GGG AAG GAG GAC GTG CAC TGC TAC AGC ATG Pro Gly Tyr Thr Thr Lys Gly Lys Glu Asp Val His Cys Tyr Ser Met 540 545 550	864
CAG AGC AAG TAG Gln Ser Lys 555	876

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 291 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met	Gln	Arg	Ala	Arg	Pro	Thr	Leu	Trp	Ala	Ala	Ala	Leu	Thr	Leu	Leu	1	5	10	15
Val	Leu	Leu	Arg	Gly	Pro	Pro	Val	Ala	Arg	Ala	Gly	Ala	Ser	Ser	Ala	20	25	30	
Gly	Leu	Gly	Pro	Val	Val	Arg	Cys	Glu	Pro	Cys	Asp	Ala	Arg	Ala	Leu	35	40	45	
Ala	Gln	Cys	Ala	Pro	Pro	Pro	Ala	Val	Cys	Ala	Glu	Leu	Val	Arg	Glu	50	55	60	
Pro	Gly	Cys	Gly	Cys	Cys	Leu	Thr	Cys	Ala	Leu	Ser	Glu	Gly	Gln	Pro	65	70	75	80
Cys	Gly	Ile	Tyr	Thr	Glu	Arg	Cys	Gly	Ser	Gly	Leu	Arg	Cys	Gln	Pro	85	90	95	
Ser	Pro	Asp	Glu	Ala	Arg	Pro	Leu	Gln	Ala	Leu	Leu	Asp	Gly	Arg	Gly	100	105	110	
Leu	Cys	Val	Asn	Ala	Ser	Ala	Val	Ser	Arg	Leu	Arg	Ala	Tyr	Leu	Leu	115	120	125	
Pro	Ala	Pro	Pro	Ala	Pro	Gly	Asn	Ala	Ser	Glu	Ser	Glu	Glu	Asp	Arg	130	135	140	
Ser	Ala	Gly	Ser	Val	Glu	Ser	Pro	Ser	Val	Ser	Ser	Thr	His	Arg	Val	145	150	155	160
Ser	Asp	Pro	Lys	Phe	His	Pro	Leu	His	Ser	Lys	Ile	Ile	Ile	Ile	Lys	165	170	175	

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Lys Gly His Ala Lys Asp Ser Gln Arg Tyr Lys Val Asp Tyr Glu Ser  
180 185 190

Gln Ser Thr Asp Thr Gln Asn Phe Ser Ser Glu Ser Lys Arg Glu Thr  
195 200 205

Glu Tyr Gly Pro Cys Arg Arg Glu Met Glu Asp Thr Leu Asn His Leu  
210 215 220

Lys Phe Leu Asn Val Leu Ser Pro Arg Gly Val His Ile Pro Asn Cys  
225 230 235 240

Asp Lys Lys Gly Phe Tyr Lys Lys Lys Gln Cys Arg Pro Ser Lys Gly  
245 250 255

Arg Lys Arg Gly Phe Cys Trp Cys Val Asp Lys Tyr Gly Gln Pro Leu  
260 265 270

Pro Gly Tyr Thr Thr Lys Gly Lys Glu Asp Val His Cys Tyr Ser Met  
275 280 285

Gln Ser Lys  
290

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## CLAIMS

1. A method for stimulating bone formation in a subject who has a bone marrow disorder causing bone loss, said method comprising administering to the subject pharmaceutically effective doses of IGF-I and IGFBP-3.
2. The method of claim 1 wherein the marrow disorder is selected from the group consisting of plasma cell dyscrasias, leukemia, lymphomas, systemic mastocytosis, anemias, lipidoses and mucopolysaccharidoses.
3. The method of claim 1 wherein a pharmaceutically effective dose of an inhibitor of bone resorption is also administered.
4. The method of claim 3, wherein the IGF-I/IGFBP and the inhibitor of bone resorption are administered sequentially.
5. The method of claim 3, wherein the inhibitor of bone resorption is an estrogen, tamoxifen, a calcitonin, a bisphosphonate or a growth factor having anti-resorptive activity.
6. The method of claim 5 wherein the growth factor is a transforming growth factor- $\beta$ .
7. The method of claim 3, wherein the treatment is prophylactic.
8. A method for stimulating bone formation in a subject who has a connective tissue disorder causing bone loss, said method comprising administering to the

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subject pharmaceutically effective doses of IGF-I and IGFBP-3.

5           9.    The method of claim 8 wherein the  
connective tissue disorder is selected from the group  
consisting of osteogenesis imperfecta, Ehlers-Danlos  
syndrome, Marfans syndrome, cutis laxa, homocystinuria,  
Mankes's syndrome and scurvy.

10           10. The method of claim 9 wherein a  
pharmaceutically effective dose of an inhibitor of bone  
resorption is also administered.

15           11. A method for stimulating bone formation in  
a subject who has drug-related osteoporosis, said method  
comprising administering to the subject pharmaceutically  
effective doses of IGF-I and IGFBP-3.

20           12. The method of claim 11 wherein the drug  
causing the osteoporosis is selected from the group  
consisting of corticosteroids, heparin, oral  
anticoagulants, anticonvulsants, methotrexate, thyroid  
hormone, lithium and gonadotrophin-releasing analogs.

25           13. The method of claim 11 wherein a  
pharmaceutically effective dose of an inhibitor of bone  
resorption is also administered.

30           14. A method for stimulating bone formation in  
a subject who has bone loss due to pregnancy, lactation,  
chronic hypophosphatemia, hyperphosphatasia, insulin-  
dependent diabetes mellitus, anorexia nervosa, cadmium  
poisoning, juvenile osteoporosis or Paget's disease of  
bone, said method comprising administering to the subject  
35   pharmaceutically effective doses of IGF-I and IGFBP-3.

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15. The method of claim 14 wherein a pharmaceutically effective dose of an inhibitor of bone resorption is also administered.

5 16. A method for stimulating bone formation in a subject who has periodontal bone loss, said method comprising administering to the subject pharmaceutically effective doses of IGF-I and IGFBP-3.

10 17. A method for stimulating bone formation in a subject who has osteoarthritis-related bone loss, said method comprising administering to the subject pharmaceutically effective doses of IGF-I and IGFBP-3.

15 18. A composition for inducing bone formation in a subject, comprising IGF-I, IGFBP and an inhibitor of bone resorption in a pharmaceutically acceptable excipient.

20 19. The composition of claim 18, wherein the inhibitor of bone resorption is an estrogen, tamoxifen, a calcitonin, a bisphosphonate or a growth factor having anti-resorptive activity.

25 20. The composition of claim 18, wherein the IGF-I or the IGFBP is conjugated to the inhibitor of bone resorption.

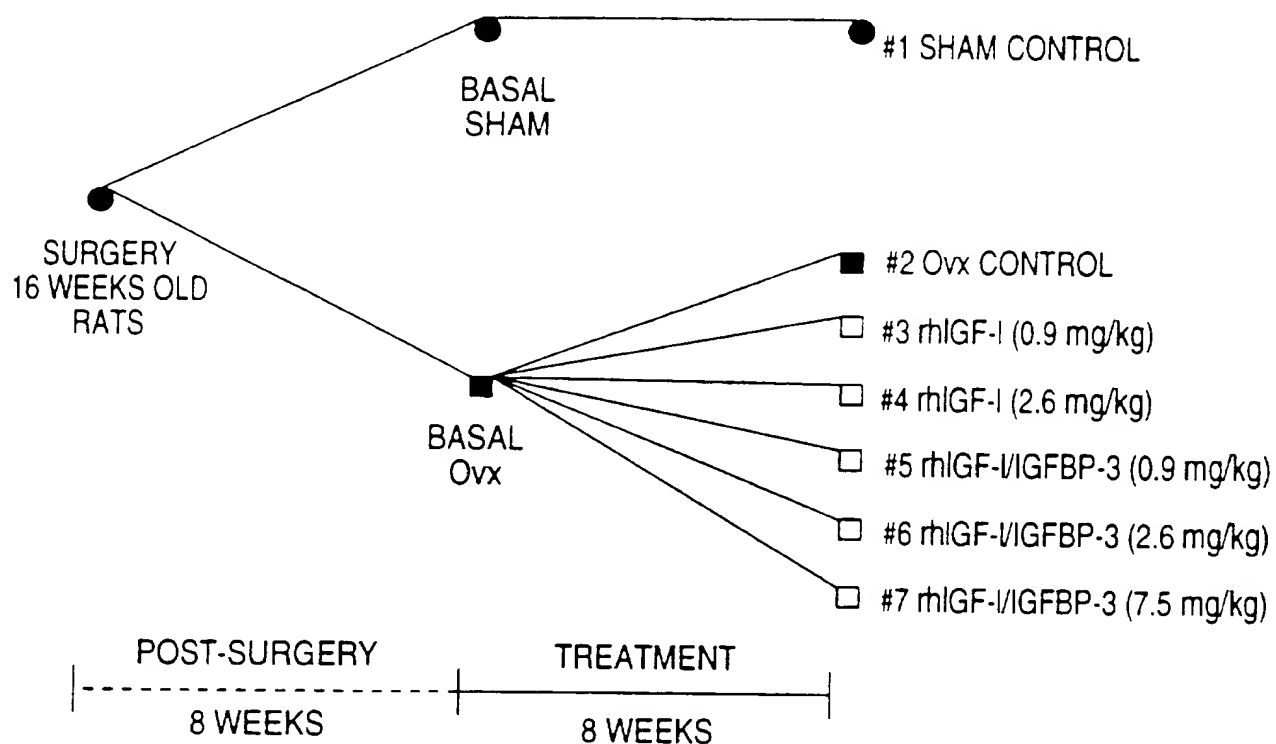
30 21. The composition of claim 18, further comprising a sustained-release vehicle.

22. The composition of claim 18, wherein the IGFBP is IGFBP-3.

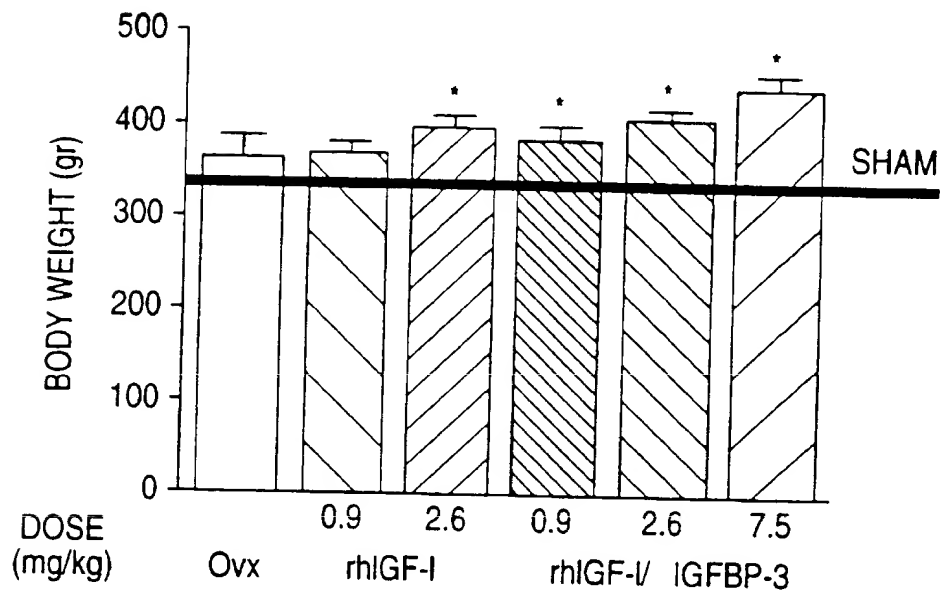
35



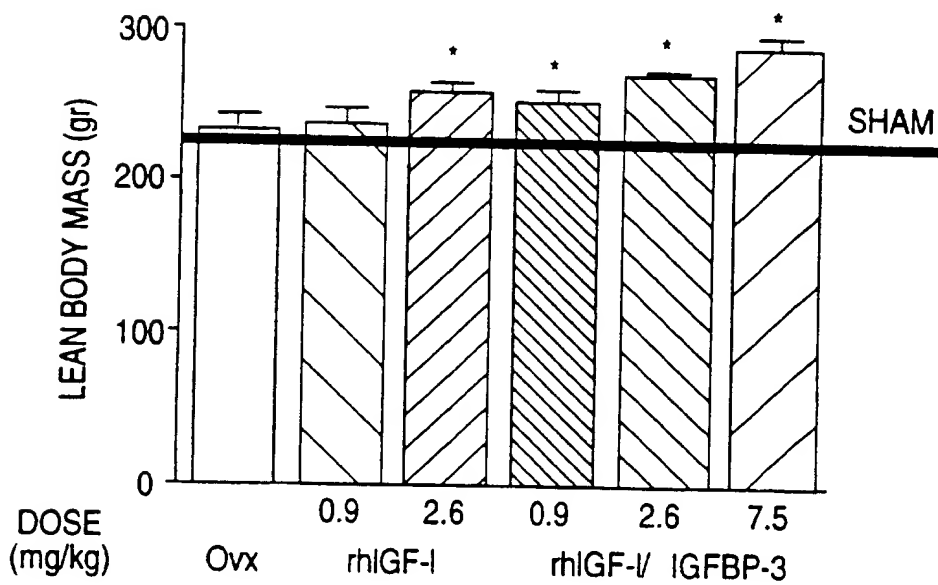
1/21

**FIG. 1**

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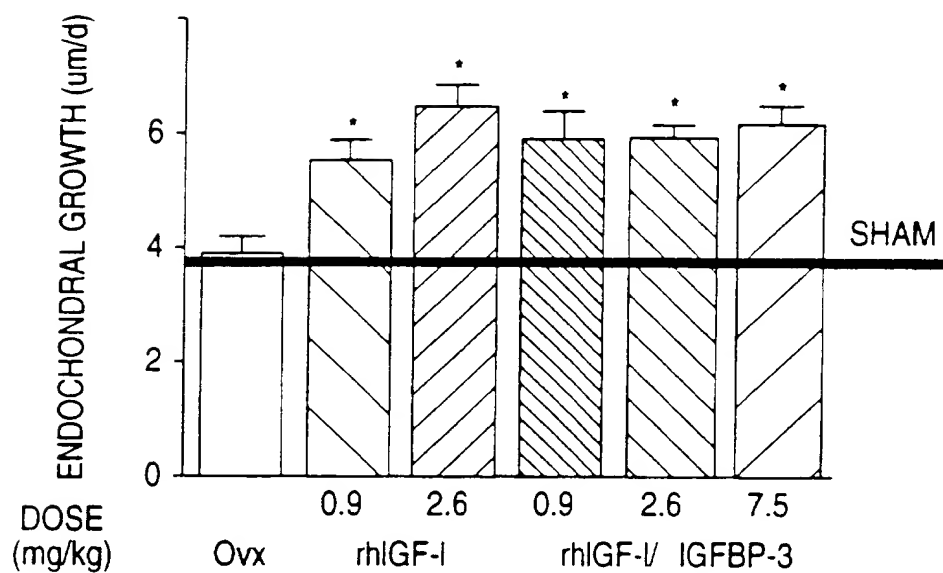
**FIG. 2A**

\*SIGNIFICANTLY DIFFERENT  
FROM Ovex GROUP

**FIG. 2B**

\*SIGNIFICANTLY DIFFERENT  
FROM Ovex GROUP

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**FIG. 3**\*SIGNIFICANTLY DIFFERENT  
FROM Ovex GROUP

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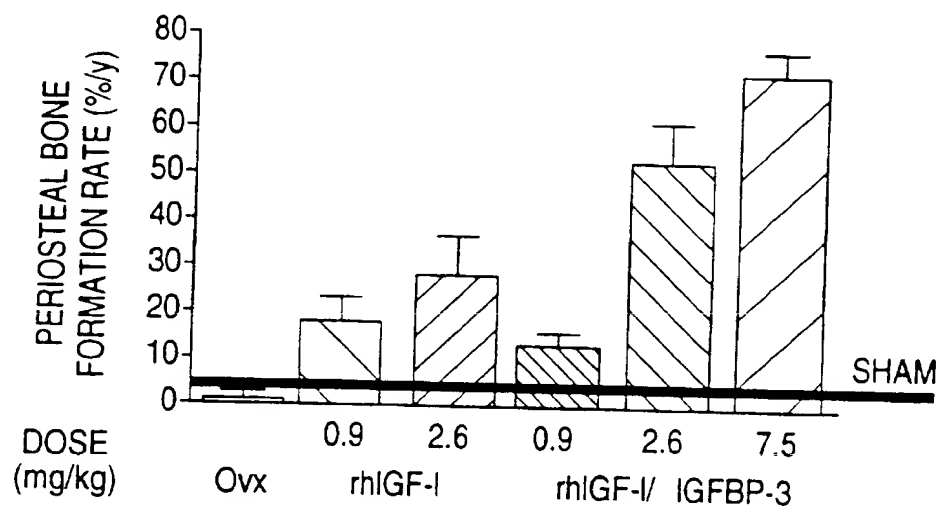


FIG. 4A

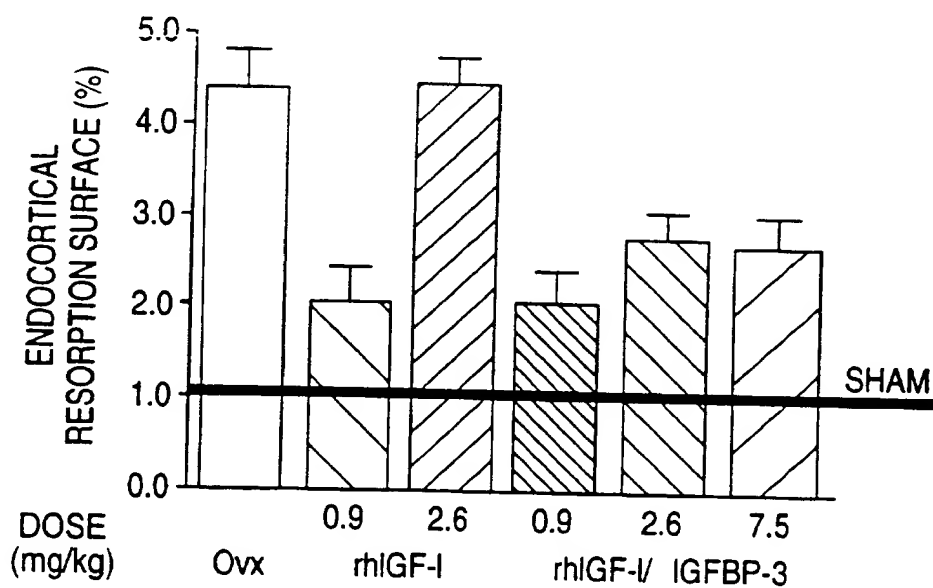


FIG. 4B

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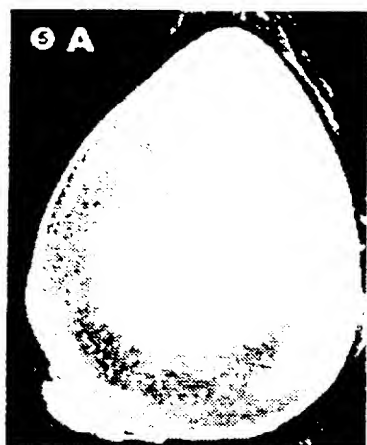


FIG. 5A

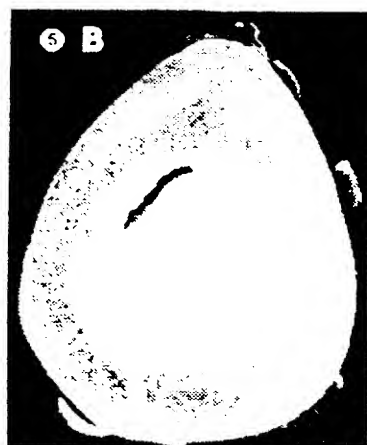


FIG. 5B

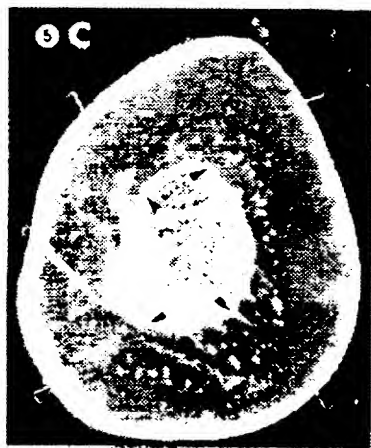


FIG. 5C



FIG. 5D

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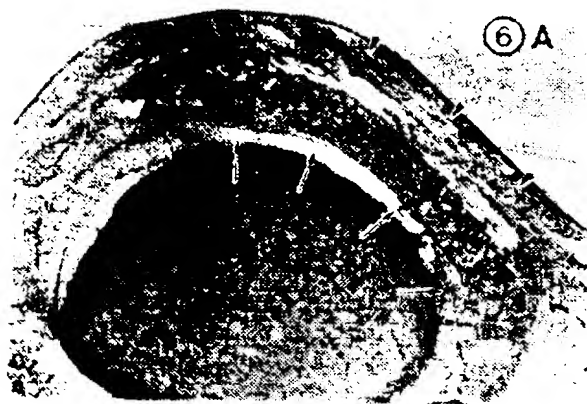


FIG. 6A

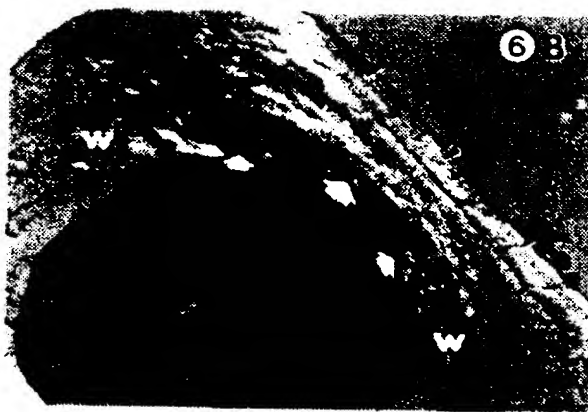


FIG. 6B

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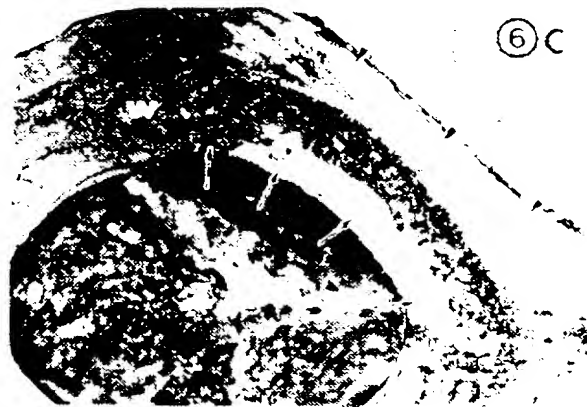


FIG. 6C

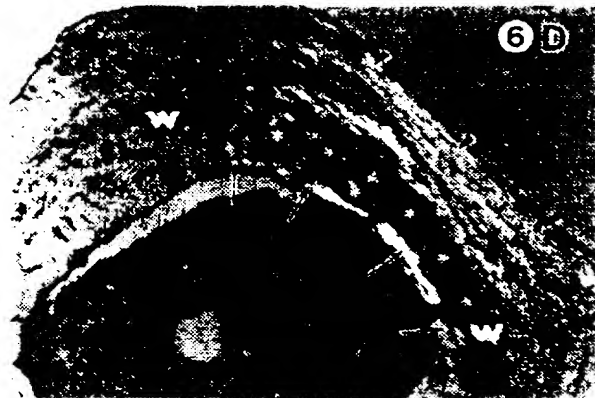


FIG. 6D

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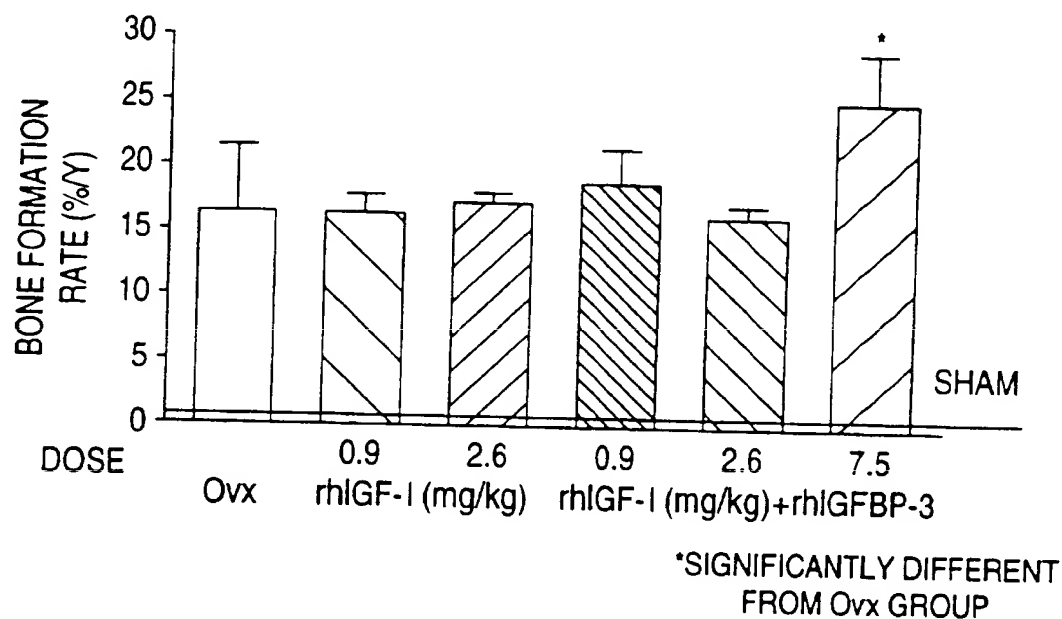


FIG. 7A

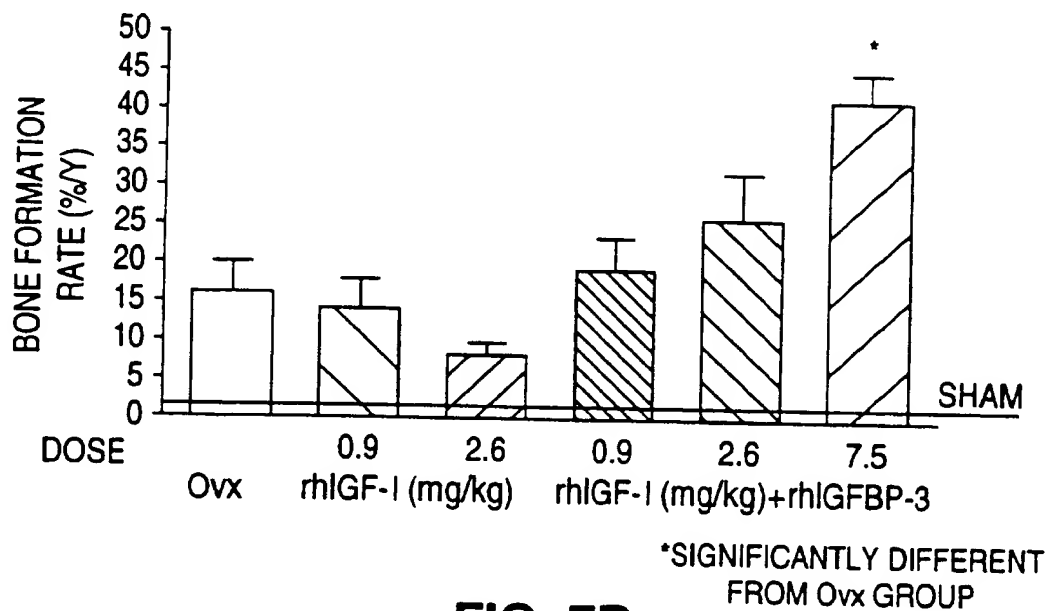
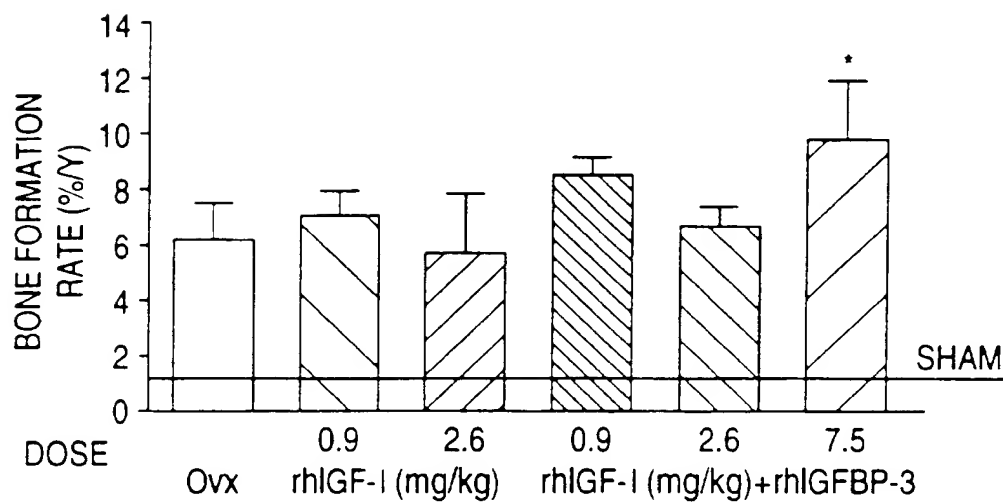
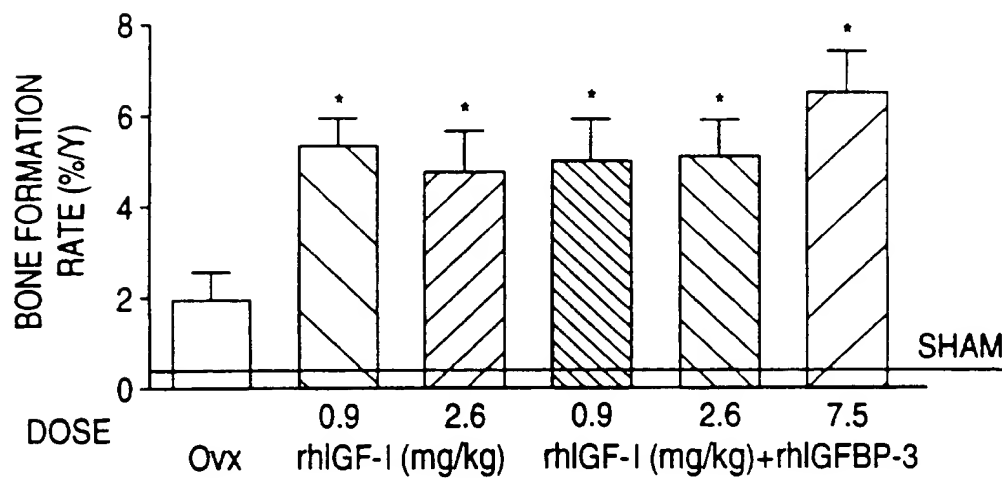


FIG. 7B



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**FIG. 7C**\*SIGNIFICANTLY DIFFERENT  
FROM Ov GROUP**FIG. 7D**\*SIGNIFICANTLY DIFFERENT  
FROM Ov GROUP

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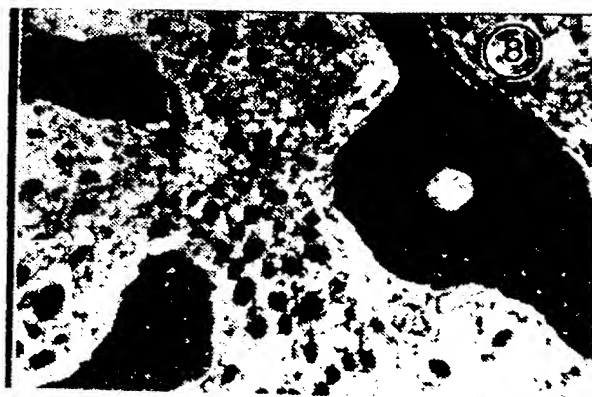


FIG. 8A

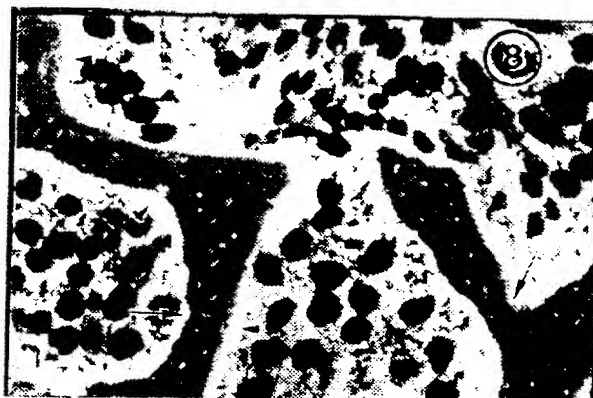


FIG. 8B

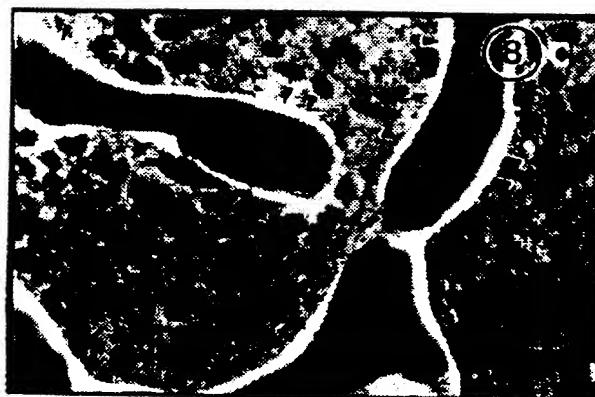
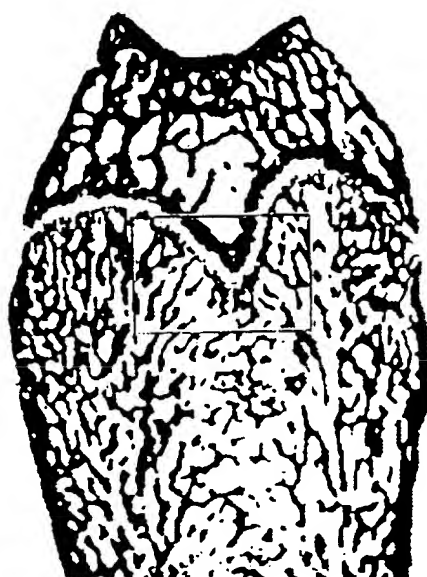


FIG. 8C

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**FIG. 9A**



**FIG. 9B**



**FIG. 9C**

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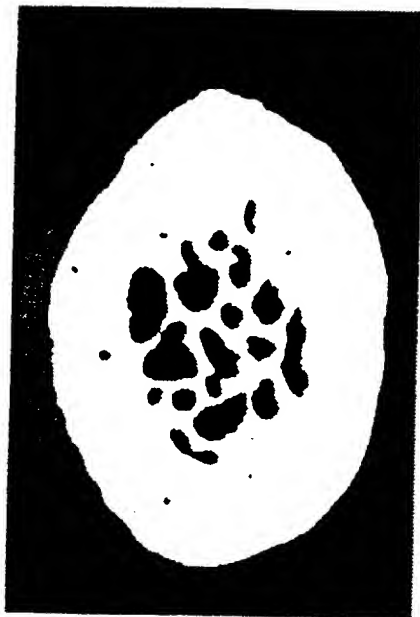


FIG. 10A



FIG. 10B



FIG. 10C

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GPETLCGAELVDALQFVCGDRGFYFNK  
PTGYGSSSRRAPQTGIVDECCFRSCDL  
RRLEMYCAPLKPAKSA

**FIG. 11**

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\*

GASSAGLGPVVRCEPCDARALAQCAPPVCAELVRE  
PGCGCCLTCAELSEGQPCGIYTERCGSGLRCQPSPDEA  
RPLQALLDGRGLCVNASAVSRLRAYLLPAPPAPGNAS  
ESEEDRSAGSVESPSVSSTHRVSDPKFHPLHSKIIII  
KKGHAKDSQRYKVDYESQSTDTONFSSESRETEYGP  
CRREMEDTLNHLKFLNVLSPRGVHIPNCDKKGFYKKK  
QCRPSKGRKRGFCWCVDKYGQPLPGYTTKGKEDVHCY  
SMQSK

\* There is a natural heterogeneity at position 5; glycine can also occur at this position.

**FIG. 12**

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156 ATG GGT GCA TCT TCT GCA GGT TTA GGT CCA GTT GTT CGT TGT GAA CCA  
 Met Gly Ala Ser Ser 5  
 1  
 204 TGT GAT GCT CGT GCT CTT GCT CAA TGT GCT CCA CCA CCA GCT GTT TGT  
 Cys Asp Ala Arg Ala Leu Ala Gln Cys Ala Pro Pro Ala Val Cys  
 20 25 30  
 252 GCT GAA CTT GTT CGT GAA CCG GGT TGT GGT TGT TGT CTG ACT TGC GCA  
 Ala Glu Leu Val 35  
 40 45  
 300 CTT TCT GAA GGT CAA CCA TGT GGT ATT TAT ACT GAA CGT TGT GGT TCT  
 Leu Ser Glu Gly Gln Pro Cys 55  
 60  
 348 GGT CTG CGT TGT CAA CCA TCT CCA GAT GAA GCT CGT CCT CTG CAG GCT  
 Gly Leu Arg Cys Gln Pro Ser Pro Asp Glu Ala Arg Pro Leu Gln Ala  
 65 70 75 80

FIG. 13A

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```

396  CTG CTG GAC GGT CGT GGT CTG TGC GTT AAC GCT TCC GCT GTT TCC CGT
      Leu Leu Asp Gly Arg Gly Leu Cys Val Asn Ala Ser Ala Val Ser Arg
      85
      90
      95

444  CTG CGC GCC TAC CTG CTG CCA GCG CCG CCA GCT CCA GGA AAT GCT AGT
      Leu Arg Ala Tyr Leu Leu Pro Ala Pro Pro Ala Pro Gly Asn Ala Ser
      100
      105
      110

492  GAG TCG GAG GAA GAC GAC CGC AGC GCC GGC AGT GTG GAG AGC CCG TCC GTC
      Glu Ser Glu Glu Asp Arg Ser Ala Ala Gly Ser Val Glu Ser Pro Ser Val
      115
      120
      125

540  TCC AGC ACG CAC CGG GTG TCT GAT CCC AAG TTC CAC CCC CTC CAT TCA
      Ser Ser Thr His Arg Val Ser Asp Pro Lys Phe His Pro Leu His Ser
      130
      135
      140

588  AAG ATA ATC ATC ATC AAG AAA GGG CAT GCT AAA GAC AGC CAG CGC TAC
      Lys Ile Ile Ile Ile Lys Lys Gly His Ala Lys Asp Ser Gln Arg Tyr
      145
      150
      155
      160

```

FIG. 13B



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636	AAA GTT GAC TAC GAG TCT CAG AGC ACA GAT ACC CAG AAC TTC TCC TCC	Lys Val Asp Tyr Glu Ser Gln Ser Thr Asp Thr Gln Asn Phe Ser Ser	165 170 175
684	GAG TCC AAG CGG GAG ACA GAA TAT GGT CCC TGC CGT AGA GAA ATG GAA	Glu Ser Lys Arg Glu Thr Glu Tyr Gly Pro Cys Arg Arg Glu Met Glu	180 185 190
732	GAC ACA CTG AAT CAC CTG AAG TTC CTC AAT GTG CTG AGT CCC AGG GGT	Asp Thr Leu Asn His Leu Lys Phe Leu Asn Val Leu Ser Pro Arg Gly	195 200 205
780	GTA CAC ATT CCC AAC TGT GAC AAG AAG GGA TTT TAT AAG AAA AAG CAG	Val His Ile Pro Asn Cys Asp Lys Lys Gly Phe Tyr Lys Lys Lys Gln	210 215 220
828	TGT CGC CCT TCC AAA GGC AGG AAG CGG GGC TTC TGC TGG TGT GTG GAT	Cys Arg Pro Ser Lys Gly Arg Lys Arg Gly Phe Cys Trp Cys Val Asp	225 230 235 240

FIG. 13C

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876	AAG	TAT	GGG	CAG	CCT	CTG	CCA	GGC	TAC	ACC	ACC	AAG	GGG	AAG	GAG	GAC
	Lys	Tyr	Gly	Gln	Pro	Leu	Pro	Gly	Tyr	Thr	Thr	Lys	Gly	Lys	Glu	Asp
					245					250					255	
924	GTG	CAC	TGC	TAC	AGC	ATG	CAG	AGC	AAG	TAG						
	Val	His	Cys	Tyr	Ser	Met	Gln	Ser	Lys							
				260					265							

FIG. 13D

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10	20	30	40	50	60
123 456 789 012 345 678 901 234 567 890 123 456 789 012 345 678 901 234 567 890					
GGT GCT TCT TCT GCT GGT CTT GGA CCA GTT GTT CGT TGT GAA CCA TGT GAT GCA CGA GCT					60
TTA GCT CAA TGT GCT CCA CCA CCA GCT GTT TGT GCT GAA TTA GTT CGA GAA CCA GGT TGT					120
GGT TGT TGT TTA ACT TGT GCT TTA TCT GAA GGT CAA CCA TGT GGT ATT TAT ACT GAA CGT					180
TGC GGT AGT GGT TTG CGT TGT CAA CCA CCA AGC CCA GAT GAA GCT AGG CCT TTA CAA GCA TTA					240
TTA GAT GGT CGA GGT CTG TGT GTT AAT GCG TCC GCT GTT TCT CGA TTG CGC GCT TAT TTA					300
TTA CCT GCC CCA CCG GCA CCG GGT AAC GCC TCC GAA AGC GAA GAG GAT CGT TCT GCG GGT					360
TCC GTT GAA TCT CCA AGT GTG AGT TCT ACC CAT CGA GTT AGC GAC CCG AAA TTT CAT CCG					420
TTG CAC TCT AAA ATC ATT ATT ATT AAA AAG GGT CAC GCA AAG GAT TCT CAA CGT TAT AAG					480
GTG GAT TAT GAA AGC CAA TCT ACC GAC ACT CAA AAT TTT AGT AGT GAA AGT AAA CGT GAA					540
ACC GAG TAC GGC CCG TGT CGA CGT GAG ATG GAG GAT ACC TTA AAC CAT TTA AAA TTT TTG					600
AAC GTT TTA TCC CCG CGT GGC GTT CAT ATC CCG AAT TGC GAT AAA AAA GGC TTC TAC AAA					660
AAG AAA CAA TGC CGT CCG AGT AAG GGT CGT AAA CGA GGT TTT TGT TGC GTT GAC AAA					720
TAC GGT CAA CCG TTG CCG GGT TAT ACT ACT AAA GGC AAA GAA GAT GTT CAT TGT TAT TCT					780
ATG CAA TCT AAA TAA TGC ATC TCG AGA ATT C					811

FIG. 14

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ATGCAGCGGGCGGACCCACGCTCTGGGCGGCTGCGCTGACTCTGCTGGTGTCTCCGCGGGCGCGGTGGCGGGGTGGC  
 MetGlnArgAlaArgProThrLeuTrpAlaAlaAlaLeuThrLeuLeuValLeuLeuArgGlyProProValAlaArgAlaGly  
 -1 +1

GCGAGCTCGGGGGCTTGGGTCCCGTGGTGGCTGCGAGCCGTGCGACGCGGTGCACTGGCCCCAGTGGCGCCCTCCGCCCGCGGTGTGGCGGGAGCTG  
 AlaSerSerAlaGlyLeuGlyProValValArgCysGluProCysAspAlaArgAlaLeuAlaGlnCysAlaProProAlaValCysAlaGluLeu

GTGCGGAGCCGGGCTGCGGCTGCTGCCTGACGTGCGCACTGAGCGAGGGCCAGCCGTGCGGCATCTACACGAGCGCTGTGGCTCCGGCCTTCGCTGC  
 ValArgGluProGlyCysGlyCysLeuThrCysAlaLeuSerGluGlyGlnProCysGlyIleTyrThrGluArgCysGlySerGlyLeuArgCys

CAGCCGTGCGCCGACGAGGCGCGCTGCAGGCGCTGCTGGACGGCGCGGGCTCTGCGTCAACGCTAGTGCCGTACGCCGCTGCGGCGCTACCTG  
 GlnProSerProAspGluAlaArgProLeuGlnAlaLeuLeuAspGlyArgGlyLeuCysValAsnAlaSerAlaValSerArgLeuArgAlaTyrLeu

CTGCCAGCGCGCAGCTCCAGGAAATGCTAGTGTGAGTCGGAGGAAGACCGCAGCGCGGCGAGTGTGGAGAGCCCGTCCGTCTCCAGCACGCACCGGGTG  
 LeuProAlaProProAlaProGlyAsnAlaSerGluSerGluSerGluSerValGlySerValGluSerProSerValSerSerThrHisArgVal

TCTGATCCCAAGTTCACCCCTCCATTCAAAGATAATCATCATCAAGAAAGGCATGCTAAAGACAGCCAGCGCTACAAAGTTGACTACGAGTCTCAG  
 SerAspProLysPheHisProLeuHisSerLysIleIleIleIleLysLysGlyHisAlaLysAspSerGlnArgTyrLysValAspTyrGluSerGln

FIG. 15A

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AGCACAGATACCCAGAACTTCTCCTCCGAGTCCAAGCGGGAGACAGAATATGGTCCCTGCCGTAGAGAAATGGAAGACACACTGAATCACCTGAAGTTC  
SerThrAspThrGlnAsnPheSerSerGluSerSerGluThrGluTyrGlyProCysArgArgGluMetGluAspThrLeuAsnHisLeuLysPhe

CTCAATGTGCTGAGTCCCAGGGGTGTACACATTCCCAACTGTGACAAGAAGGGATTATTAAGAAAAAGCAGTGTGCCCTTCCAAAGGCAGGAAGCGG  
LeuAsnValLeuSerProArgGlyValHisIleProAsnCysAspLysLysGlyPheTyrLysLysLysGlnCysArgProSerLysGlyArgLysArg

GGCTTCTGCTGGTGTGGATAAGTATGGGCAGCCTCTCCCAGGCTACACCACCAAGGGGAAGGACGTGCACGTGCTACAGCATGCAGAGCAAGTAG  
GlyPheCysTrpCysValAspLysTyrGlyGlnProLeuProGlyTyrThrThrLysGlyLysGluAspValHisCysTyrSerMetGlnSerLys.

FIG. 15B

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/08925

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) Please See Extra Sheet

US CL 514/12, 21

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. 514/12, 21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Spencer, "Modern Concepts of Insulin-like Growth Factors," published 1991 by Elsevier Science Publishing Co., Inc. (New York, NY), pages 381-383, see entire document.	1-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*I* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

07 SEPTEMBER 1995

Date of mailing of the international search report

17 OCT 1995

Name and mailing address of the ISA/US  
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Facsimile No. (703) 305-3230

Authorized officer

P. Lynn Touzeau, Ph.D.

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No  
PCT/US95/08925

A CLASSIFICATION OF SUBJECT MATTER  
IPC (6)

C07K 14/00, 14/65; A61K 38/00, 38/28

